metric techniques
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MANOMETRIC TECHNIQUES AND RELATED METHODS FOR THE STUDY OF TISSUE METABOLISM

by

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Chapters on Specialized Techniques

by

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INTRODUCTION

This book is an effort to provide the beginning graduate student with a simple working description of the methods which are more or less routinely used in the study of tissue metabolism. Because of the remarkable progress which has been made during the past decade, a working knowledge of such methods is today a part of the training in almost any field of biology. We have thus attempted to bring together the basic and most widely used techniques for the study of tissue metabolism. We have designed this as a laboratory volume written for the beginner.

In a sense, it is the laboratory companion to the earlier "Respiratory Enzymes" but it grew out of a somewhat different need and has a somewhat different purpose. We have written this volume for our own students. In it we have attempted to provide a workable series of methods which they would probably find useful in their own studies. This viewpoint has imposed three limitations on the methods included, as follows:

1. We have included only those methods with which we ourselves have worked and which we have found to be satisfactory.
2. We have included only methods which require a minimum of equipment such as is likely to be found in a laboratory engaged in biological research. The chief items required are a respirometer, a colorimeter, and a centrifuge. With the methods we have attempted to give the working details, the results of our experience with them, and certain precautions, hints, etc. which are sometimes necessary when even a perfectly adequate method is used by the novice.
3. We have included a wide range of methods and approaches in addition to those involving manometric measurements. Especially we have provided some information on the character, preparation, and estimation of the phosphorylated intermediates.

We recognize that the methods described may not be the best ones available. They are, however, usually quite adequate; they can be executed with relatively little equipment, and they have been very useful in our laboratories. We regard this book as a start in placing in the hands of the beginning graduate student sound basic methods which will permit him to successfully carry out important studies. We would welcome descriptions of techniques which we have inadvertantly omitted, improvements of those described, or other suggestions which might improve this book's usefulness. We would hope to include these in any later editions of this volume which may appear.

A word should be said about the manner in which this book was written. The authors originally prepared some of the material for use in their own classes with no thought of the present volume. When its preparation was begun, a considerable body of information had already been accumulated. Drs. Cohen, LePage, and Potter generously contributed chapters on methods employed in their laboratories. Others prepared smaller sections on certain specialized techniques. These sections were left largely in words of the contributor and incorporated into the work at suitable points by the authors. Because of the existing situation some of the contributors have had no opportunity to see the work as a whole, or even their section of it in final form. Portions not definitely assigned to an individual were prepared jointly by the authors. We are indebted to each of the contributors for their wholehearted cooperation, to Drs. Barber and Bartholomew who prepared sections which were eventually omitted, to many others who offered suggestions but were unable, because of present circumstances, to prepare detailed descriptions of the methods, to the American Instrument Co. for permismission to reproduce figs. 2, 11, 14, 24, 25, and 30, to the A. H. Thomas Co. for fig. 20, to the Central Scientific Co. for fig. 21, and to E. Machlett and Son for fig. 26b.

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Chapter One

THE WARBURG CONSTANT VOLUME RESPIROMETER

W. W. Umbreit

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Chapter One

GENERAL PRINCIPLES OF THE WARBURG CONSTANT VOLUME RESPIROMETER AND ITS APPLICATION TO THE MEASUREMENT OF OXYGEN UPTAKE

INTRODUCTION

Ma[illegible]etric methods for estimating exchange of gases have been in use in the study of oth ch[illegible]cal and biological reactions for generations. A wide variety of techniques have een em[illegible]yed and many types of apparatus have been developed. The type of respirometer hich h[illegible] met with widest use is almost universally known as the "Warburg" instrument lthoug[illegible] as pointed out by Warburg (1926) it has a long history. In essence, the present nstrum[illegible]is a modification of a "blood-gas manometer" described by Barcroft and Haldane 1902). [illegible]e essential principle involved is that if, at constant temperature, one holds he vol[illegible]of a gas constant, any changes in the amount of gas can be measured by changes n pressure. This method is most commonly applied to measurements of oxygen uptake. We herefo[illegible]irst describe its principles in terms of oxygen uptake and later consider other ıses t[illegible]ch the instrument may be applied.

APPARATUS

F = flask
S = sidearm
G = sidearm stopper with gas vent
C = center well (for alkali)
M = manometer proper
R = fluid reservoir which, by adjustment of the screw clamp, serves to alter the level of the fluid in the manometer
T = three-way stopcock

The scale of the manometer is graduated in centimeters (numbered) and in millimeters. Normally one records readings in terms of millimeters.

The apparatus consists of a flask (F) (fig. 1) (detachable) sometimes equipped with one or more sidearms (S), attached to a manometer (M) containing a liquid of known density. The flask is immersed in a water bath at a constant temperature, and between readings the system is shaken or whirled to promote a rapid gas exchange between the fluid and the gas phase. Details of the apparatus have been described by Burk and Milner (1932), Dixon (1943), Perkins (1943), Warburg (1923, 1924, 1926) and others.

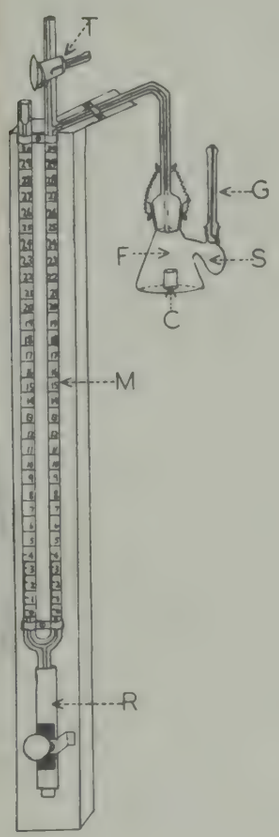

Fig. 1. The Warburg constant volume manometer.

Fig. 2 illustrates such a bath with its attendant apparatus for maintaining constant temperatures and for shaking a bank (usually 7 to a side) of such manometers.

The manometer has (as shown in fig. 1) an open and a closed end. A given point on the closed side of the manometer (usually 15 or 25 cm.) is chosen, and the liquid in the closed arm of the manometer is always adjusted to this point before recording pressure changes.

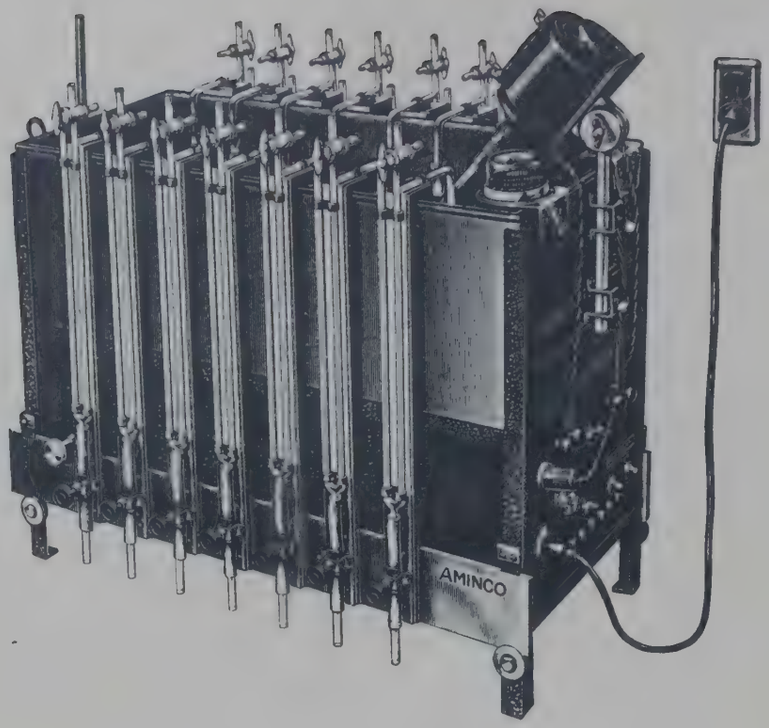

Fig. 2. Constant temperature bath with manometers.

GENERAL PRINCIPLES

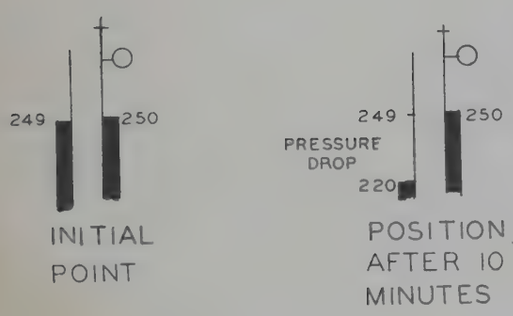

Fig. 3. Diagram illustrating the determination of pressure change.

Suppose that one has a reaction going on in the flask which is using up oxygen. One adjusts the closed side of the manometer (with stopcock open) to 250 mm. (by adjusting the screw clamp on the fluid reservoir of the manometer), closes the stopcock, and reads the open side of the manometer. (Compare diagram, fig. 3). Suppose that the level of the liquid in this arm is 249 mm. This reading of 249 is recorded. After 10 minutes time the liquid has gone up in the closed arm and down in the open arm. One again adjusts the closed arm to 250 mm. (thus holding the volume of gas in the flask constant and reading a changing pressure). The reading on the open arm is now 220 mm. Since, initially, when the closed arm was at 250, the reading on the open arm was 249 and now, when the closed arm is at 250, the reading is 220, there has been a drop in pressure of from 249 to 220 (or 29 mm.) in the flask due to the oxygen used. If one knows the gas volume of the flask (V_g), the volume of fluid in the flask (V_f), the temperature of operation, the gas being exchanged and the density of the fluid in the manometer, it is possible to calculate the amount of gas used up (or given off), providing only one gas is being changed and one knows which gas it is. There are methods for handling alterations in the amount of more than one gas. These will be described later. The essence of the method is to hold the gas and fluid volumes constant and measure the decrease or increase in pressure when one gas alters in amount.

DERIVATION AND MEANING OF FLASK CONSTANT

Fundamentally this consists of so calibrating the system that from the observed pressure changes one can calculate the amount (in $mm.^3$ or micro liters (µl) at 0°C. and 760 mm pressure) of gas utilized or given off.

The following symbols are employed:

Let h = the observed change in the manometer (open end) reading in mm.
x = µl. gas (0°C., 760 mm. pressure).
V_g = Volume of gas phase in flask (including connecting tubes down to the zero point (15 or 25 cm. on closed end of manometer).

V_f = Volume of fluid in vessel.

P = Initial pressure in vessel of the gas involved in the determination. This is actually the partial pressure of the particular gas in a gas mixture. If this gas mixture contains water vapor, the partial pressure of the gas involved in the determination will be less than its partial pressure in the dry condition. Hence if P is defined as dry gas, P - R should be used in equations involving moist gases.

P_o = 760 mm. (Hg, standard pressure) expressed in terms of the manometer fluid:

P_o = 760 x 13.60 (Density of mercury)/Density of manometer fluid.

T = Temperature of bath in absolute degrees (= 273 + T°C.).

α = Solubility of gas involved in liquid in vessel (expressed as ml. gas/ml. liquid when gas is at a pressure of one atmosphere (760 mm. Hg) at the temperature T).

R = Vapor pressure of water (or other fluid) at temperature T. Inside the flask one has both fluid and gas. This fluid will exert a vapor pressure (R) in the gas phase and some gas will dissolve in the fluid.

In the gas phase one has gas (V_g) at a temperature (T) and at a pressure P - R (= partial pressure of the gas involved less the vapor pressure). Since one can change

$$PV/T = P'V'/T'$$

this gas volume to standard conditions (let prime symbols be standard conditions, i.e. V' = gas volume standard conditions, P' = P_o = 760 mm. Hg, T' = 273 = 0°C.). Hence in the flask:

$$(P - R)\, V_g/T = P_o\, V'/273 \text{ or}$$

$$\text{gas at standard conditions} = V' = \frac{V_g \frac{273}{T} (P-R)}{P_o}$$

In the fluid at the start one has some gas dissolved. The amount of gas in the fluid is:

$$V_f\, \alpha (P - R)/(P_o)$$

Where α is the solubility of the gas (in ml./ml.) at a partial pressure of one atmosphere. The $(P - R)/P_o$ converts the solubility at one atmosphere to that actually existing in the flask.

This relationship holds since, from Henry's law; "The concentration of dissolved gas is directly proportional to the concentration (pressure) above the fluid." Hence if α is the solubility at P_o (one atmosphere) the solubility at the actual pressure existing in the flask (P - R) (atmospheric pressure less than that due to water vapor) will be $\frac{P - R}{P_o}$. Virtually nothing is known of the relationship between chemical structure and solubility of gases so that one has to determine the solubility empirically. There is thus a different solubility for each gas in each solution. It is known, however, that the solubility of individual gases in a mixture is almost independent of the pressure of others, i.e., the solubility of oxygen at a given pressure and at a given temperature will be the same whether N_2, CO_2 or other gases are present or not.

From the considerations above, the gas present at the start was that in the gas phase and that in the fluid phase or:

$$\text{Gas at start} = \underbrace{V_g \frac{273}{T} \frac{(P - R)}{P_o}}_{\text{Gas phase}} + \underbrace{V_f\, \alpha \frac{(P - R)}{P_o}}_{\text{Fluid phase}}$$

At the end of the observation period this gas has been changed by the amount x which has resulted in a pressu change of h mm. If gas is taken up, h is negative; if gas is given off, h is positive. We will here assume that it is taken up. The pressure is now (P - R - h) rather than (P - R) initially.

Gas phase is thus: $V_g \frac{273}{T} \frac{(P - R - h)}{P_o}$

Liquid phase: $V_f \alpha \frac{(P - R - h)}{P_o}$

Gas at end = $V_g \frac{273}{T} \frac{(P - R - h)}{P_o} + V_f \alpha \frac{(P - R - h)}{P_o}$

Gas taken up (x) is that which was present initially less that which appears at the end.

x = initial gas - final gas

$$= \left[V_g \frac{273}{T} \frac{(P - R)}{P_o} + V_f \alpha \frac{(P - R)}{P_o}\right] - \left[V_g \frac{273}{T} \frac{(P - R - h)}{P_o} + V_f \alpha \frac{P - R - h}{P_o}\right]$$

$$= V_g \frac{273}{T} \frac{(P - R)}{P_o} + V_f \alpha \frac{(P - R)}{P_o} - V_g \frac{273}{T} \frac{(P - R - h)}{P_o} - V_f \alpha \frac{P - R - h}{P_o}$$

$$= V_g \frac{273}{T} \frac{h}{P_o} + V_f \alpha \frac{h}{P_o}$$

$$x = h \left[\frac{V_g \frac{273}{T} + V_f \alpha}{P_o}\right] = h\,k$$

(Flask constant)

Note that V_g, T, α, V_f and P_o are known and, for a given experiment, are constant; hence by determining this constant one can convert mm. pressure change into µl. O_2 taken up.

Summary:

x = amount of gas exchanged = h (alteration in reading on open arm of manometer) k (flask constant)

$$k = \text{flask constant} = \frac{V_g \frac{273}{T} + V_f \alpha}{P_o}$$

Example: A Warburg flask has a total volume of 12.616 ml. up to the 250 mm. mark on the manometer. To measure oxygen uptake in this flask in a yeast suspension at 28°C, we add 2 ml. of yeast, 1 ml. of 0.1 M glucose, 1 ml. of M/50 phosphate buffer. In the center cup of the flask we place 0.2 ml. 10% KOH to absorb the carbon dioxide the yeast may produce. What flask constant should be employed?

V_f = 3.2 ml. = 3,200 µl.

V_g = total volume - fluid volume = 12.616 ml. - 3.2 ml. = 9.416 ml. = 9,416 µl.

T = 273 + 28 = 301 α = 0.027 P_o = 10010

$$k_{O_2} = \frac{V_g \frac{273}{T} + V_f \alpha}{P_o} = \frac{9{,}416 \times \frac{273}{301} + 3200 \times 0.027}{10010} = \frac{8340 + 86.0}{10010} = 0.841$$

Difference in pressure x 0.841 = µl. gas
(in mm.)

(Chapter Six describes other methods of determining the flask constants)

Two points may be confusing. One, the choice of 0.027 for α, will be explained in the next section. The other is the use of P_o = 10010. The manometer in this case was filled with "Brodie's Solution" composed as follows:

23 gram NaCl
5 gram Sodium Choleate (Merck)
in 500 cc. water
Usually colored with dye.

This has a density of 1.030 so that

$$P_o = 760 \times \frac{13.60}{1.030} = 10010$$

THE SOLUBILITY OF OXYGEN

This is expressed in the table (Table I) below as ml. O_2/ml. fluid when the gas is at one atmosphere pressure ($=\alpha$). This term is sometimes referred to as the "Bunsen Coefficient."

Two aspects of the gas laws are of interest. The first, apparent from the α values cited, is that the solubility of gases is decreased as temperature rises. The other is that the solubility of gases is "appreciably" diminished by the presence of dissolved solids (or liquids, but not gases) in the fluid. This is thought to be due to the hydration ("solvation") of the solute; i.e. less free solvent is thus available for dissolving the gas. These effects are shown in table II constructed from data in the International Critical Tables, Volume III, p. 271. (1928).

TABLE I

The Solubility of Oxygen

Data as ml. gas dissolved per ml. fluid
when gas is at 1 atmosphere pressure (α value)

TEMPERATURE C	RINGER'S SOLUTION	WATER
0		0.04872
10	0.0480	0.03793
15	0.0340	0.03441
20	0.0310	0.03091
25	0.0285	0.02822
30	0.0260	0.02612
35	0.0245	
40	0.0230	
	DIXON (1943)	International Critical Tables (1928)

TABLE II

The Influence of Salts Upon the Solubility of Oxygen

Data in terms of ml. O_2 dissolved per ml. solution (α value)

	HCl, 15°C	HCl, 25°C	1/2 H_2SO_4 25°C	NaCl 25°C
Conc	0.034	0.028	0.028	0.028
0.5 M	0.033	0.027	0.027	0.024
1.0 M	0.031	0.026	0.025	0.020
2.0 M	0.028	0.025	0.023	0.016

While the effect of salts on oxygen solubility appears large, it actually makes little difference to the Warburg system. For example, a change of from pure water to 2 M NaCl, changes the α value from 0.028 to 0.016. This alters the flask constant by $\frac{0.012 \times V_f}{10010}$. In the case described above, the k instead of being 0.841 would be, with 2 M NaCl, 0.838.

THE THERMOBAROMETER

In the development of the flask constant, k, a value P was employed which was thought to remain constant from the beginning to the end of a given period. This "P" represented the initial atmospheric pressure. The pressure in the room and the temperature of the water bath are likely to change, however, and these changes are corrected for by a thermobarometer. The thermobarometer consists merely of a Warburg manometer with a flask containing water attached; the volume of water is not critical.

Referring first to fig. 4, "initial", which represents the start of the reading. The respiring flask reads 249, the thermobarometer 250. At the end of a given period the respiring flask has dropped to 220, (i.e., 29 mm. While the thermobarometer, due solely to the increased pressure in the room, has dropped to 248 (i.e., 2 mm.). The decrease in pressure observed in the respiring flask was due to two things: the use of some of the oxygen in the gas space inside the flask (27 mm.) and the increase of gas pressure in the room (2 mm.). Corrections of the readings obtained for changes in pressure in the room are obvious after a brief study of the following table which illustrates actual data obtained. (Table III)

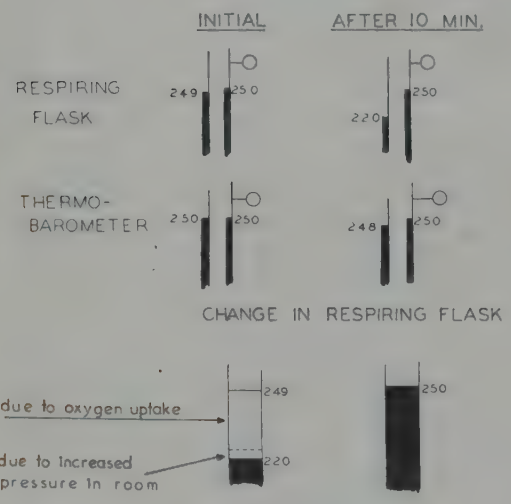

Fig. 4. Diagrams illustrating the use of the thermobarometer.

If the level of liquid in the thermobarometer has risen, this means that the external pressure in the room has decreased. For reaction flasks which have dropped, the observed decrease is smaller than the actual decrease by the amount of the pressure change in the room; hence the rise in the thermobarometer is added to the observed pressure drop. If the level of liquid in the reaction flask has risen, the thermobarometer rise is subtracted from the rise observed. These are illustrated in the examples given in Tables III and IV

TABLE III

Corrections for Thermobarometer Changes

Time	Thermobarometer		Method 1 Respiring Flask #1			Method 2 Respiring Flask #1			
		Change (total)	Reading	Change	True Change	Reading	Chg. Interval	True Chg. Interval	Sum
0	250 mm.		249	0	0	249			
60 min.	257 mm. (+7)	+7	248	-1	-8	248	-1	-8	-8
120 min.	259 mm. (+2)	+9	243	-6	-15	243	-5	-7	-15
0	250	-	249	-					
60	236	-14	111	-138	-124	-	-	-	-

SAMPLE CALCULATIONS

The data of Table IV illustrate the method of calculating the µl. oxygen uptake from the observed changes in the level of the manometer fluid in the thermobarometer and in the reaction flask. Two methods of such calculation are noted as follows:

Total uptake method:

Columns 4, 5, 6 and 7 of Table IV. The uptake in mm. is calculated by subtracting the initial reading (246) from all subsequent readings (column 4). The thermobarometer correction is obtained by subtracting the initial reading (265) from all subsequent readings (column 5). Since in the interval from 10^{55} to 11^{00} the total uptake of 19 mm. in the reaction flask was due in part (1 mm.) to the increased pressure on the outside, the real uptake was 19 - 1 = 18 mm. (column 6). This value times the flask constant for the conditions employed gives the µl. oxygen taken up (column 7).

Interval uptake method:

In this method each reading is subtracted from the one following it (i.e., 246 from 227; 227 from 194, etc.) giving the uptake (column 8) over the interval. A similar calculation is made for the thermobarometer (column 9) from which the actual uptake (column 10) is readily apparent. These interval values are multiplied by the flask constant (column 11) to yield the uptake per interval and are added to yield the total uptake (column 12).

While this method appears more laborious, it offers some advantages. Especially when the rate of oxygen uptake is changing. In this case, for instance, the uptake during the first five minutes (17.3 µl.) is not the same as in the succeeding 5 minute intervals; in fact, there is a tendency for the rate to increase throughout the determinations. This increase is not readily apparent when calculated by the "total method" and may even be overlooked in graphing.

Many laboratories have found it convenient to employ mimeographed charts for recording the readings and thermobarometer corrections. The information recorded in the permanent notebook is the uptake value corrected for the thermobarometer change.

TEMPERATURE CONTROLS

How accurately must the temperature of the bath be controlled? We are here dealing with two situations as follows:

I. The whole bath is at a constant temperature but has dropped to 1° below that accepted for calculation. The thermobarometer has responded to this drop in temperature so the main error involved is due to using the wrong factor. Suppose that in the case described in Table IV, the temperature throughout the bath dropped to 27°C while the data is being calculated as at 28°. At 28° the k_{O_2} was 0.942; at 27° it would be 0.945. If the measurements were actually made at 27°C, but the factor for 28°C was used, roughly a 0.3% error is involved.

II. If the bath were not uniform, however, a difference in temperature of 1°C from one part of the bath to the other would cause a change of 33 µl. per 10 ml. gas volume of a flask (difference of 0.05°C = 1.7 µl.).

Hence two factors are important; one, that the temperature be held at the point desired and two, that the temperature of the entire bath be held uniform to within 0.05°C. This latter factor necessitates vigorous sti[illegible]

TABLE IV

Calculating Oxygen Uptake from Manometer Readings

Time	Reading Thermo-barometer	Reading flask	Total method				Interval method			
			Uptake in mm.	Thermo-barometer correction	Actual uptake in mm.	µl. O_2 uptake	uptake in mm.	Thermo-barometer correction	Actual uptake in mm.	µl. O_2 uptake
	mm.	mm.								
10^{55}	265	246	-	-	-	-	-	-	-	-
11^{00}	264	227	-19	-1	-18	17.0	-19	-1	-18	17.0
11^{05}	264	194	-52	-1	-51	48.1	-33	0	-33	31.1
11^{10}	264	159	-87	-1	-86	81.0	-35	0	-35	32.9
11^{15}	264 (2)	122 (3)	-124 (4)	-1 (5)	-123 (6)	114.9 (7)	-37 (8)	0 (9)	-37 (10)	34.9 (11)

(1)

Flask: 1 ml. yeast suspension, 1 ml. M/200 KH_2PO_4, pH 4.5, 0.5 ml. water, 0.5 ml. 0.032 M glucose; glucose i

sidearm, tipped in at 10^{55}.

Volume flask - 13.5 ml. k_{O_2} = 0.942 Temp. 28°C.; 0.2 ml. KOH in center cup.

THE USE OF THE WARBURG INSTRUMENT FOR THE MEASUREMENT OF RESPIRATION OF LIVING CELLS

Physiologically there are two meanings for the word "respiration". The older meaning confines the term to the actual uptake of gaseous oxygen. It was later realized that oxidations could occur (by the removal of hydrogen or electrons) without employing gaseous oxygen and so the term respiration was broadened to include any reaction by which the cell obtained energy, whether or not it involved gaseous oxygen as such. This has resulted in some confusion since the meaning of the term thus differs with different groups of investigators. For the purposes of this outline the following definitions are employed:

Respiration: The uptake of gaseous oxygen.
Fermentation: The transformations which occur in living cells (or enzymes therefrom) which do not employ gaseous oxygen.

In the case of most cells, as contrasted to many enzyme preparations, the utilization of oxygen results in a release of carbon dioxide. If these two gases (CO_2, O_2) are the only ones involved, one can measure the respiration (O_2 uptake) by absorbing the liberated carbon dioxide in alkali. In the presence of alkali the carbon dioxide pressure in the air is zero within the limits of measurement. The gas exchange caused by the respiration is oxygen absorption plus carbon dioxide liberation. But the alkali keeps the carbon dioxide pressure zero, hence the change noted on the barometer is due solely to the oxygen utilization. The excess of carbon dioxide in solution, of course, continually distills over into the alkali, but it does not affect the pressure changes.

THE ABSORPTION OF OXYGEN

The absorption of oxygen by the respiring tissue takes place almost entirely from the oxygen in solution. This is the principle reason for shaking the fluids in the respirometer, i.e. to obtain a fluid phase saturated with the gas phase. But one must, under practical circumstances, take care that the rate of oxygen uptake by the tissue is not greater than can be replaced by the diffusion of oxygen from the atmosphere into the fluid. If the rate of oxygen uptake is so high that the oxygen is used up faster than it can diffuse into the liquid, then the rate of respiration is dependent upon the rate at which oxygen diffuses into the fluid and has little to do with the potential rate of the reaction itself.

The rate at which gas diffuses into a liquid is dependent upon the surface layer of the liquid. The gas may be thought of as moving across a film of surface, and the theory of such diffusion has been well worked out. Roughton (1941) has described methods for correcting for diffusion errors when they exist. But for the purpose of virtually all respiratory measurements it is sufficient to note that by shaking the flasks one obtains a continual new surface exposed to the gas by virtue of the turbulence of the fluid in the flask. Hence, the greater the rate of shaking, the greater the rate of diffusion of the gas into the liquid, and the greater the rate of respiration one may measure without diffusion errors.

Dixon and Tunnicliffe (1923) and Dixon and Elliott (1930) have studied these effects in the Barcroft differential manometer (see Chapter 7) and concluded that 600-700 $\mu l.O_2$ per hour could be safely measured without diffusion errors when a shaking rate of 100 oscillations per minute were employed (over 1500 $\mu l.O_2$/hr. at a rate of 138 oscillations per minute).

In the Warburg respirometer the surface exposed to the gas is less than in the Barcroft type, hence limiting rates of oxygen uptake are reached sooner. The actual rates possible for flasks approximately 15 ml. in volume containing 3 ml. of fluid have been determined in the experiment described below which illustrates one method of ensuring that the rate of oxygen diffusion is not the limiting factor in any results one might obtain (Fig. 5).

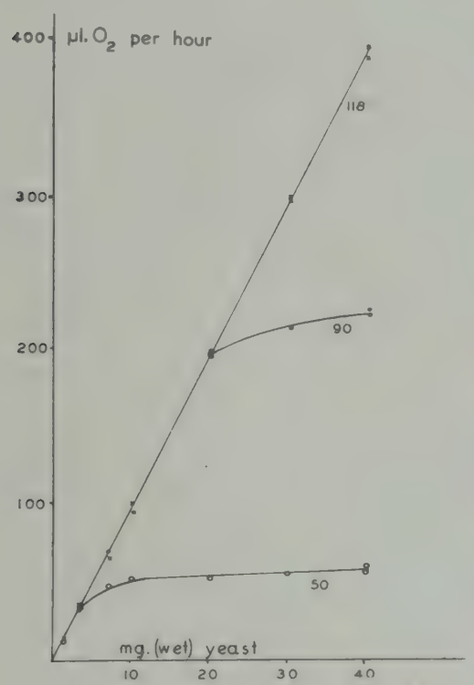

Fig. 5. Influence of shaking on rate of oxygen uptake. Warburg flasks of about 15 ml. capacity. Each flask has amount of yeast indicated made to 2 ml. with M/50 KH_2PO_4 (pH 4.8), 1 ml. of 3% glucose and 0.2 ml. 20% KOH in center well. Shaken at 50, 90 and 118 two-centimeter strokes per minute at 28°C.

The basis of the experiment described is as follows: the rate of oxygen diffusion from the gas phase into the fluid phase is dependent upon the surface boundary. This boundary is altered more rapidly with faster shaking allowing more rapid oxygen exchange. If more rapid shaking (and thus more rapid oxygen exchange) does not increase the rate of oxygen uptake, then the rate of oxygen diffusion is not the limiting factor in the respiration being studied.

Another principle which may be used (but only in some circumstances) is that the higher the concentration of the gas, the greater will be its rate of diffusion into a liquid. Hence, one could increase the oxygen in the atmosphere above the liquid, using increasing quantities of tissue, and determine the maximum rate of oxygen uptake that one could employ before diffusion factors were significant. This method is, however, not only more laborious, but also more complex in that there are some types of respiration which are dependent upon the pressure of oxygen. Therefore changing the shaking rate is the preferred method. Increasing the oxygen pressure is useful, however, when one finds it necessary to supply adequate concentrations of oxygen throughout a solid tissue. In this case the diffusion into the liquid is not the limiting factor, but diffusion into the respiring solid controls the oxygen level at its center. Obviously increased shaking will not alter these surfaces, so that the only practical solution is

to increase the oxygen pressure. This is discussed under "tissue slices" in Chapter 8.

It sometimes happens that a reaction is dependent upon a contact between particles and that shaking disturbs this contact. One such example reported is the sulfur oxidation by bacteria (Vogler, LePage and Umbreit, 1942) in which a contact between the bacteria and the solid sulfur particles is necessary before oxidation can occur (Vogler and Umbreit, 1941, Umbreit, Vogel and Vogler, 1942). Shaking at rapid rates actually disturbs such contact and results in lowered oxidation. However, it is notable that if one employs rates of oxygen uptake lower than those at which diffusion becomes significant (i.e., at 100 strokes per minute, 300 µl.O_2/hr.) any variation in the rate of oxygen uptake with increase in shaking rate is not dependent upon the diffusion of oxygen since the fluid is already saturated. Therefore it is always desirable to determine the effect of alterations in the shaking rate to be certain that the results are independent of the rate of shaking. If they are not, one can readily determine whether the shaking rate is affecting the diffusion of oxygen or other factors (such as contact) by comparison with the rate of oxygen uptake which could be measured without diffusion effects under the conditions employed. Frequently important clues as to the nature of the reactions involved are obtained in this way.

THE ABSORPTION OF CARBON DIOXIDE

In studying the oxygen uptake by living tissues which also liberate CO_2 using the "Direct Method" of Warburg the CO_2 is absorbed in alkali, i.e. one keeps the pressure of CO_2 in the gas phase at zero throughout the determinations. If, because of the failure of the alkali employed to completely and instantaneously absorb the CO_2, the CO_2 pressure in the gas phase is not zero, the readings on the manometer will not represent oxygen uptake. An example of circumstances of this type is given in the report of Brock, Druckrey, and Richter (1936) in which, because of the large amounts of CO_2 liberated, readings on the manometer dropped only slightly or in some instances actually rose, yet oxygen was being consumed at a rapid rate. These workers found that the absorption of CO_2 was instantaneous and that its pressure was readily held at zero if the rate of CO_2 liberation was not more than 600 µl. per hour. Dixon and Elliot (1930) found that in the Barcroft apparatus (in the presence of adequate surface, see below) 1000 µl. of CO_2 per hour was instantaneously absorbed.

In absorbing CO_2 from the gas phase the same difficulties are encountered as with the absorption of oxygen. Here, however, because the alkali is usually confined to the small center cup, an increased rate of shaking has little effect in increasing surface. Hence some other method must be employed to increase the surface of the alkali. Usually small rolls of filter paper are placed in the alkali cup. These should project beyond the side walls of the center cup into the open gas space above. A desirable projection is about 5 mm. Such "KOH papers" are usually prepared in quantity by cutting filter paper into squares with 2 cm. sides (the exact dimensions vary with the depth of the cup employed; this varies from instrument to instrument, but the size need be only approximate). These papers are then folded, three or four times, accordion fashion, and inserted into the center cup with tweezers. When wet by the alkali, previously added to the cup, they spring open and provide a large surface for the absorption of CO_2.

Sufficient alkali should be added to moisten the paper entirely and still leave a well of free liquid in the bottom of the cup. For the papers described 0.2 ml. is adequate. Sometimes difficulty is experienced in that the alkali tends to "creep over" the cup into the outer compartment of the flask. This can be prevented by greasing the top of the cup before inserting the papers. A convenient way of doing this is to wind a small amount of cotton about the end of a glass rod so that when placed over the center cup it will completely cover it. By saturating the cotton with grease, the top surface of the center cup can be given a light coat of grease with ease (also see Chapter 6).

The concentration of alkali employed by various investigators differs widely but KOH (because of the solubility of the potassium carbonate) is almost universally employed. Two things must be kept in mind in choosing the concentration. One is the capacity of the alkali employed; the other is the ease with which the alkali is handled. While the pressure of CO_2 above any solution of KOH is zero, at very dilute solutions of the latter the alkali may be completely neutralized very soon. Under most circumstances 1% KOH is undoubtedly sufficient. Most workers use either 5, 10 or 20% KOH to be sure that an

adequate supply is present to last throughout the experiment. 20% KOH offers no difficulty in handling. It is claimed by some that rather concentrated solutions of KOH (10-20%) react with the filter papers employed and that an oxygen uptake results from this reaction. While we have never experienced this reaction, the recommendation that analytical grade filter papers be used for KOH papers should be followed whenever possible.

It is obvious that the conditions for obtaining adequate oxygen diffusion and CO_2 absorption are easily met. Usually the shaking rates employed are 100 two centimeter strokes per minute. Under these conditions (employing flasks of about 15 ml. capacity) one must use amounts of tissues that take up less than 300 µl. of O_2 per hour and give off less than 500 µl. CO_2. This usually means about 100 mg. (wet weight) of animal tissues, somewhat less for yeast and bacteria. For the beginner it is well to choose tissue concentrations which take up about 200 µl. O_2 per hour.

PROCEDURE EMPLOYED

The actual procedure in setting up systems for the measurement of respiration of living cells varies quite widely. A common procedure is listed as follows:

1. To clean, dry, Warburg flasks equipped with a center well, add materials (except cells) to the main compartment of flask.
2. Add materials (if any) to the sidearm.
3. Add 0.2 cc alkali (usually 5, 10 or 20% KOH).
4. Grease attachment joint on manometer and grease and insert plug for sidearm. Grease top of alkali cup if desirable.
5. Add cells.
6. Add filter paper strip to alkali in center cup (see absorption of carbon dioxide).
7. Attach flask to manometer.
8. Place in constant temperature bath.
9. Adjust and tighten flask after about 5 min. shaking in bath. (This is done since sometimes the grease becomes softer and the flask tends to creep slightly)
10. Allow to equilibriate, with shaking, for 10-15 minutes.
11. Adjust manometer fluid to zero point (closed side of manometer with stopcock open).
12. Close stopcock.
13. Begin readings.

LIMITATIONS OF METHOD

The method described in the previous sections, whereby any carbon dioxide formed is absorbed by alkali, is known as Warburg's "Direct Method". It is the method most widely used for determining respiration. As with any other method it has certain limitations. These are:

1. The gases exchanged must be only O_2 and CO_2. In most cases this condition is not difficult to meet since in the majority of tissues these are the only gases involved. Warburg (1926), however, points out that "the metabolism of bacteria is rarely so simple that it can be measured by this method". This is a somewhat pessimistic viewpoint, and many bacteria can be studied adequately by this method. However, one should always take care to check that the only gases involved are O_2 and CO_2 before relying upon data derived by this method.
2. One must work in an atmosphere free from carbon dioxide. For some tissues, this is of no consequence, i.e. they respire at the same rate, to the same extent, and follow the same pathways whether CO_2 be present or not. But for others, this is by no means true. Carbon dioxide may inhibit, may stimulate, or may alter the path of metabolism of a given cell, hence measurements in the absence of CO_2 may not give a reasonable estimate of the reactions occurring in its presence. For this purpose the Warburg "Indirect method" may be used (see Chapter 4).
3. The rate of oxygen uptake, and the rate of carbon dioxide liberation and absorption must be within a particular range so that the assumptions of the method hold, i.e. that the fluid is always saturated with oxygen gas (or air) and that the pressure of carbon dioxide in the gas phase is always zero.

Thus, in spite of the limitations of the "Direct Method" it does find wide application and only in rare cases does it yield inaccurate results. The conditions necessary for adequate functioning can be met with ease in most cases.

RESULTS OF DETERMINATIONS

The Warburg "Direct Method" is capable of two general types of use:

1. The determination of the rate of oxygen uptake.
2. The determination of the amount of oxygen uptake.

Both are usually measurable in the same determination. In expressing the rate of oxygen uptake, a quotient ("Q") is commonly employed. Several of these are in general use. These are defined as follows:

Q_{O_2} = µl. O_2 taken up per mg. dry weight of tissue per hour.

$Q_{O_2}(N)$ = µl.O_2 taken up per mg. tissue nitrogen per hour. (Earlier written qO_2)

$Q_{O_2}(P)$ = µl.O_2 taken up per mg. tissue phosphorus per hour, or per mg. nucleic acid phosphorus per hour.

$Q_{O_2}(C)$ = µl.O_2 taken up per mg. tissue carbon per hour.

$Q_{O_2}(cell)$ = µl.O_2 taken up per cell per hour.

In short, one specifies in the Q term the conditions under which the rate was measured and the basis which one used to estimate the amount of tissue. In a general way:

$$Q \begin{matrix} (\text{gas atmosphere}) \\ (\text{gas measured}) \end{matrix} (\text{tissue basis})$$

For example:

$Q^{O_2}_{O_2}(N)$ means µl.O_2 taken up per mg. nitrogen of tissue per hour in an atmosphere of pure oxygen.

$Q^{N_2}_{CO_2}(P)$ means µl.CO_2 given off in an atmosphere of nitrogen (or under anaerobic conditions) per mg. of tissue phosphorus per hour.

Uptake of a gas is indicated by a minus (-) sign, release by a plus (+). Two conventions are employed:

1. When the gas atmosphere is air, the condition indicator is omitted.
2. When the tissue basis is dry weight, this indicator is omitted.

Thus, the term Q_{O_2} is used rather than $Q^{air}_{O_2}(\text{dry weight})$, and $Q_{O_2}(N)$ means oxygen uptake in air per unit nitrogen per hour, while $Q^{O_2}_{O_2}(N)$ means oxygen uptake in pure oxygen per unit nitrogen per hour.

The varied values which are employed (Q_{O_2} and $Q_{O_2}(N)$ being the most common) represent an effort to estimate the "active" portion of the cell constituents. For example, many bacteria produce a "gum", a carbohydrate material on the cell surface. This material is not "alive" (being usually a reserve carbohydrate that can be metabolized but slowly), yet it contributes to the cell volume, the wet weight, the dry weight, and, because it frequently occludes mineral matter from the medium, it even contributes to the ash. Because of this gum formation, the dry weight, etc. of such cells does not actually measure the cell content. One then attempts to obtain a convenient measure of the amount

of the living portion of the cell, usually nitrogen or phosphorus, or some other factor which is not influenced by the mere accumulation of an "inert" storage product. The actual measure one does employ is dependent upon the tissue one is using and it should be one closely connected with cell activity. For a further discussion see Burris and Wilson (1940) and Berenblum, Chain and Heatley (1939). Care must be taken, in comparing respiration rates (Q_{O_2} values) from tissues of different sorts, that the presence of inert materials has not contributed to the basis used and thus influenced the rate obtained.

One can also compare the effect of the treatments without knowing the exact amount of tissue involved, as long as it is the same in all cases. Thus, one might use 1 ml. of a bacterial suspension (whose dry weight, nitrogen, etc. were not known) in each of a series of buffers at different pH's. One could compare the effect of pH among the flasks employed without determining the exact amount of tissue.

The second use to which the "Direct Method" may be put is the determination of the amount of oxygen taken up per unit of substrate added, i.e. how many moles of oxygen are used in oxidizing x moles of substrate. For this purpose one usually employs flasks with sidearms. A known quantity of the material to be studied is placed in the sidearm. After equilibration, the rate of oxygen uptake is determined in the absence of substrate (to be certain that it is constant), the substrate is tipped in, and the oxygen uptake determined until it again reaches the endogenous (respiration in the absence of substrate) rate. An example of this type of data is given in an experiment shown in fig. 6.

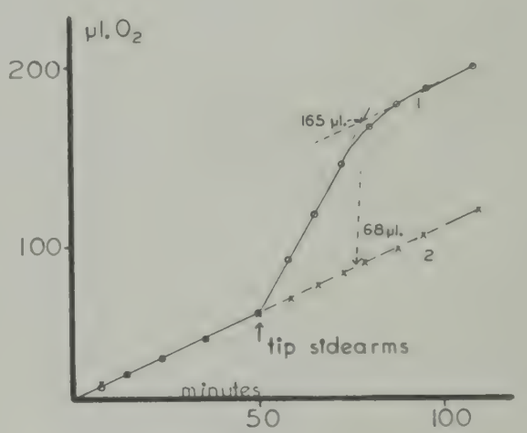

Fig. 6. Example of the quantitative use of the Warburg Direct Method. Flasks contain: center; 1 ml. yeast (containing 79.5 micrograms yeast nitrogen), 0.5 ml. M/100 KH_2PO_4 (pH 4.8), 0.5 ml. water. Sidearm: Flask 1: 0.1 ml. glucose (5.0 mg. glucose per ml.), 0.25 ml. M/100 KH_2PO_4 (so that the phosphate and potassium concentrations do not change on tipping), 0.65 ml. water. Flask 2: 0.25 ml. M/100 KH_2PO_4, 0.75 ml. water. Each flask contains 0.2 ml. 20% KOH in center cup. From flask 1, 0.5 mg. glucose added = 0.5/180 x 1000 = 2.78 µM 2.78 µM x 22.4 = 62.2 µl. glucose added. Observed 97 µl. O_2 consumed due to glucose addition (165 µl - 68 µl, see graph), or 97/62.2 = 1.56 O_2 per glucose. Since complete oxidation of glucose to CO_2 and water would require 6 O_2 per molecule, 1.56/6 = 26.0% of the glucose was completely oxidized.

It will be noted that from this type of experiment it is possible to obtain both the amount of oxygen per mole of substrate and the rate of oxygen uptake. For example, in the experiment cited, the rate of oxygen uptake can be calculated from the period of 50 to 80 minutes; (Q_{O_2}(N) = 1710). The rate obtained under these conditions may not, however, be the maximum rate possible, since, in order to measure the oxygen taken up in a reasonable length of time, one may find it necessary to add substrate in quantities insufficient to saturate the enzyme systems. Normally the enzyme systems are considered saturated if one obtains a straight line function with time, but occasionally instances may be found in which the rate of oxygen uptake (or other functions of metabolism) may proceed in a linear manner, yet higher levels of the substrate will increase the rate.

In determining the amount of oxygen taken up per unit of substrate, it is frequently something of a problem to decide whether one should subtract from the oxygen uptake observed in the presence of substrate, the oxygen taken up over the same interval in the absence of substrate. That is, when a substrate is being oxidized at a rapid rate, does the endogenous respiration continue at its constant rate, or is it suppressed, or does it increase? These questions have not yet been answered. Undoubtedly they depend upon the tissue involved, and no general answer can be given. However, it is always good practice to determine the endogenous respiration and to report it, along with the oxidation in the presence of substrate, and to indicate whether or not the endogenous respiration was substracted from the substrate respiration in calculating the oxygen consumption per mole of substrate. See Van Niel (1943) for further discussion.

It is frequently desirable to express concentration of substrate employed in terms of gas produced or absorbed. Since 1 mole of any gas (at standard conditions) occupies 22.4 liters, it is possible to speak of any substance in terms of liters, if each mole = 22.4 liters. The following table (Table V) makes this clear. One may thus speak of adding 11.2 µl. of glucose, which means that one has added 0.5 ml. of 0.001 M glucose solution or 5×10^{-7} moles of glucose.

TABLE V

Relations between Concentration and Microliters

Unit	Contained In	Gas Volume	µl.
1 mole	1 liter of a 1 molar solution	22.4 liters	2.24×10^7
1 millimole	1 ml. of a 1 molar solution	22.4 ml.	2.24×10^4
0.1 "	1 ml. of a 0.1 M solution	2.24 ml.	2.24×10^3
0.01 "	1 ml. of a 0.01 M solution	0.224 ml.	224
1 micromole	1 ml. of a 0.001 M solution	0.022 ml.	22.4

It is also possible to reverse this procedure and to determine µM of oxygen consumed rather than µl. One need only divide the µl. of O_2 consumed by 22.4 to obtain micromoles (10^{-3} millimoles) of the gas used. It is sometimes even convenient to employ a "Molar Flask Constant" rather than the usual flask constant such that the readings on the manometer may be directly converted into micromoles consumed. To obtain the "Molar Flask Constant" one need only divide the usual flask constant by 22.4. It should be noted that what is measured on the manometer is O_2, not O, i.e. its molecular weight is 32.

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Chapter Two

THE "DIRECT METHOD" OF MEASURING CARBON DIOXIDE OUTPUT IN RESPIRATION

W. W. Umbreit

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Chapter Two

THE "DIRECT METHOD" OF MEASURING CARBON DIOXIDE OUTPUT IN RESPIRATION

THEORY

In the previous chapter we noted that one could measure respiration even if carbon dioxide were given off, by absorbing all of the carbon dioxide in alkali. Therefore, if one had two flasks each respiring in exactly the same way, except that in one, the carbon dioxide was absorbed whereas in the other it was not, one would have a measure of the carbon dioxide liberated (Dixon, 1943; Warburg, 1926). There is one precaution which must be noted; one tissue (absence of alkali) is respiring in the presence of carbon dioxide, the other (with alkali) respires in its absence. If this should make a difference in the rate of oxygen uptake, or indeed in the course of the reactions followed, an error would be introduced. In most cases carbon dioxide has little effect upon the rate of respiration and methods will be described later (Chapter IV) which enable one to determine whether carbon dioxide is influencing the respiration rate and even to measure its rate under these conditions.

Using the symbols employed in Chapter 1, the change in the manometer fluid level (h) read on the flask without alkali, is the resultant of two forces, one, the absorption of oxygen, the other the liberation of carbon dioxide.

Manometer change due to oxygen absorption = $h_{O_2} = x_{O_2}/k_{O_2}$ since $x_{O_2} = h_{O_2}k_{O_2}$ (the formula for the flask constant)

Manometer change due to carbon dioxide production = $h_{CO_2} = x_{CO_2}/k_{CO_2}$

The final observed reading h in the flask without KOH, would be the resultant of the two, i.e.

$$h = h_{O_2} + h_{CO_2} = x_{O_2}/k_{O_2} + x_{CO_2}/k_{CO_2}$$

hence

$$(1)\ x_{CO_2} = (h - x_{O_2}/k_{O_2})\ k_{CO_2}\ (x_{O_2} \text{ obtained from flask with KOH})$$

Now x_{O_2} is known from the manometer which contained KOH, and k_{O_2} and k_{CO_2} are known for flask 2, hence X_{CO_2} can be calculated.

Example: 1 ml. of an algal cell suspension was placed in each of two flasks together with 2 ml. of water. Flask 1 (k_{O_2} = 0.96) contained 0.2 ml. KOH, flask 2 (k_{O_2} = 1.04, k_{CO_2} = 1.25) had no KOH. After equilibrating, respiration was permitted for 30 minutes. In flask 1, the change in reading (h) was 28 mm., hence, 28 x 0.96 = 26.9 µl. O_2 taken up.

In flask 2, over the same interval, the manometer dropped 9.5 mm. hence,

$$x_{CO_2} = (-9.5 - (-26.9)/1.04)\ 1.25 = (-9.5 + 25.8)\ 1.25 = 16.3 \times 1.25 = 20.4\ \mu l.CO_2$$

The R. Q. (Respiratory Quotient = CO_2 produced/O_2 consumed) is in this case, 20.4/26.9 = 0.76.

In essence this method determines the oxygen uptake in the absence of CO_2. One then calculates what the change in reading should have been in the other flask if no CO_2 were produced. The uptake observed is always less than this amount, hence the difference is due to CO_2 liberation.

A convenient way of recording data and making the calculations is to use a chart of approximately the following construction (Table VI).

TABLE VI

Method of Calculating Results

Time	Flask with KOH		Flask without KOH				
	h	$\mu l.O_2$	h	$\frac{x_{O_2}}{k_{O_2}}$	diff.	$\mu l.CO_2$	R. Q.
	mm. observed uptake corrected for thermobarometer	corrected h times k_{O_2} for flask	mm. observed reading corrected for thermobarometer	Column 3 divided by k_{O_2} of this flask	subtract column 5 from column 4	multiply column 6 by k_{CO_2}	divide column 7 by column 3
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
30 (from example cited)	-28	-26.9 k_{O_2}=0.96	-9.5	-25.8 k_{O_2}=1.04	+16.3	+20.4 k_{CO_2}=1.25	0.76

One may also substitute in equation (1) as follows. Equation (1) is:

(1) $x_{CO_2} = (h - x_{O_2}/k_{O_2})k_{CO_2}$, the constants referring to the flask without KOH

$x_{O_2} = h'k'_{O_2}$ where h' and k'_{O_2} refer to the flask with KOH, hence

(2) $x_{CO_2} = (h - h'k'_{O_2}/k_{O_2})k_{CO_2}$

Therefore one may calculate the amount of CO_2 released or absorbed during a given interval by subtracting from the change in reading on the flask with no alkali, the change in reading on the flask with the alkali times the ratio of the two oxygen constants, the whole times the CO_2 constant.

CORRECTIONS FOR UNEQUAL AMOUNTS OF TISSUE

It sometimes happens, in using plant or animal tissues, that one does not provide exactly the same amount of tissue to each of the flasks. If the differences in amount of tissue are not large, one may merely divide the readings (h) of each flask by the weight (or other measure of active tissue), to obtain the uptake per unit of tissue. The readings obtained with the two flasks are thus comparable. Suppose that in the example listed, the flask with KOH contained 0.97 mg. algae whereas the flask without KOH contained 1.15 mg. algae. In flask 1, the change was 28 mm., or the change per mg. of tissue was $\frac{28}{0.97} = 28.9$; O_2 uptake was thus 28.9 x 0.96 = 27.7 $\mu l.O_2$ taken up per mg. tissue. In the flask without KOH the manometer dropped 9.5 mm.; 9.5/1.15 = 8.25 mm. per mg. of tissue. The two changes are now comparable since both are based on the same quantity of tissue, hence

$x_{CO_2} = (- 8.25 - (-27.7)/1.04)\ 1.25 = (- 8.25 + 26.6)\ 1.25 = 18.35 \times 1.25$

$= 22.9\ \mu l.CO_2$ produced. R. Q. = 22.9/27.7 = 0.83

CORRECTIONS FOR RETENTION IN BUFFERS

When buffers are present they react with CO_2, for example:

$$Na_2HPO_4 + CO_2 + H_2O \rightleftharpoons NaH_2PO_4 + NaHCO_3$$

Hence the CO_2 which may escape to the air (and be measured as x_{CO_2}) may be less than that actually produced in the intervals measured. As will be shown in Chapter 3, at a

pH of 5 or below no appreciable amount of HCO^- exists, hence if the solution is adjusted at the end of the reaction to a pH of 5 or below, all such "bound CO_2" will be released. Therefore in order to obtain the total CO_2 liberated in the presence of buffers, one tips in acid from the sidearm and releases the CO_2 from the buffer. Since the tissue or the buffer may have contained bound carbon dioxide initially, three manometers are used as follows:

(1) + KOH to determine x_{O_2} (h)

(2) - KOH + acid added at end (h_2)

(3) - KOH + acid added at start (h_3)

The h_3 represents the initial bound CO_2, while h_2 represents that initially bound + that released during the respiration. One can obtain the actual CO_2 evolved as follows:

Initial bound $CO_2 = h_3k_3$

Initial bound CO_2 + CO_2 evolved = h_2k_2

Hence, CO_2 evolved is $h_2k_2 - h_3k_3$

Thus, if one takes the readings after the acid is added, the h of equation (1) is not h_2 but $h_2 - h_3 \frac{k_{3CO_2}}{k_{2CO_2}}$. Hence the equation (1) becomes $x_{CO_2} = \left[h_2 - h_3 \frac{k_{3CO_2}}{k_{2CO_2}} - \frac{x_{O_2}}{k_{2O_2}}\right] k_{2CO_2}$

A very useful method of correcting for CO_2 retention has been suggested by M. J. Johnson. Since at high pH values HCO_3^- is held in solution as well as CO_2, the effective value of α (which will be designated as α') will be larger than the true α value. Since pK'_a from the apparent first dissociation constant for carbonic acid is 6.343 (MacInnes and Belcher, 1933),

$$\frac{\alpha'}{\alpha} = \frac{(HCO_3^-) + (CO_2)}{(CO_2)} = \left[\text{antilog } (pH - 6.343)\right] + 1$$

where the pH is that in the reaction flask during the experiment. If the flask constant used is calculated by the use of α' instead of α, retention of CO_2 will be corrected for automatically. As α' increases rapidly with pH, the retention correction becomes very large at pH values above 7, and the accuracy of the CO_2 measurement suffers accordingly.

RESPIRATORY QUOTIENTS

These quotients are defined as relation between CO_2 produced/oxygen consumed and serve to indicate the nature of the metabolism. While an R. Q. of 1 would occur with carbohydrate on complete oxidation (0.9 for most proteins, 0.8 for most fats) the finding of these values does not thereby prove that metabolism of carbohydrate, protein, etc., is the cause of the R. Q. Nevertheless, the R. Q. is a most valuable index of the processes occurring and should be measured if possible. Later sections (especially Chapter 11) will describe other methods of determining this value.

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Chapter Three

CARBON DIOXIDE AND BICARBONATE

W. W. Umbreit

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Chapter Three

CARBON DIOXIDE AND BICARBONATE

THE SOLUBILITY OF CARBON DIOXIDE

The solubility of carbon dioxide in pure water is in essence no different from the solubility of other gases. While it is true that the carbon dioxide forms carbonic acid which dissociates to form H^+ and HCO_3^- in accordance with equation (3),

$$(3)\quad CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

it is also true that over 99% of carbon dioxide in solution is in the form of dissolved carbon dioxide and less than 1% exists as H_2CO_3, H^+ or HCO_3^-. In the absence of materials which can combine with the acid, the solubility is comparable to that of any other gas. There are two factors which render the actual figures obtained on solubility in pure water somewhat more variable than those for other gases. One is the somewhat higher Van der Waals forces which exist in carbon dioxide, which is equivalent to saying that carbon dioxide deviates from the laws of an ideal gas somewhat more than other gases, but the error involved in manometric work is negligible. The other factor is that in dissolving in pure water, H^+ ions are generated so that the pH does not remain at 7 but gradually decreases as the pressure of carbon dioxide is increased. The α values given in Table VII reflect both of these factors.

TABLE VII

The Solubility of Carbon Dioxide in Pure Water

Data in terms of α = ml.CO_2/ml. or µl.CO_2/µl. at one atmosphere.

Temp. °C			
0	1.713		
10	1.194	1.194	
15	1.019	1.019	1.014
20	0.878	0.878	
25	0.759		0.756
30	0.665	0.66	
35	0.592		
40	0.530	0.53	
	(1)	(2)	(3)

(1) Handbook of Chemistry and Physics (1944)
(2) Dixon (1943)
(3) International Critical Tables (1928)

THE INFLUENCE OF SALTS ON CO_2 SOLUBILITY

As was shown in the case of oxygen, the presence of other salts, etc. in solution has little effect upon the solubility of carbon dioxide, within physiological concentrations, providing that these do not combine with the carbonic acid. As is shown in Table VIII, the effect of various salts, while greater than with oxygen is largely negligible.

THE INFLUENCE OF CARBONATE AND BICARBONATE

If, however, there is anything in the solution which will combine with the carbonic acid (or bicarbonate ion), the entire solubility changes. Since from equation (3):

$$(3)\quad CO_2\ (gas) \rightleftharpoons CO_2\ (dissolved) \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

and since H_2CO_3 is essentially dependent upon CO_2 (dissolved) which is, in turn, directly dependent upon the pressure of carbon dioxide (pCO_2) in the gas phase, the equilibrium

TABLE VIII

The Influence of Salts and Other Materials upon the Solubility of Carbon Dioxide

Material	At 15°C (α values)			At 25°C (α values)		
	0.5 M	1.0 M	2.0 M	0.5 M	1.0 M	2.0 M
None	1.014	--	--	0.756	--	--
HCl	0.989	0.974	0.948	0.738	0.732	0.728
1/2 H_2SO_4	0.965	0.927	0.867	0.727	0.705	0.669
HNO_3	1.022	1.029	1.043	0.770	0.781	0.803
KCl	0.925	0.850	--	0.695	0.641	--
NH_4Cl	--	--	--	0.720	0.692	0.648
Glycerol	--	0.934	--	--	--	--

From International Critical Tables (1928)

constant of the dissociation of the carbonic acid, which would normally be written as equation (4a), becomes, in reality equation (4):

(4a) $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$ $\qquad K_1' = \dfrac{(H^+)(HCO_3^-)}{(H_2CO_3)}$

K_1' indicates the first dissociation constant of carbonic acid.

(4) $K_1 = \dfrac{(H^+)(HCO_3^-)}{(CO_2)}$

One may solve this equation (4) for H^+ as follows:

$$(H^+) = \frac{K_1(CO_2)}{(HCO_3^-)}$$

If one takes the logarithms of both sides:

$$\log (H^+) = \log \frac{K_1(CO_2)}{(HCO_3^-)}$$

But a -log (H^+) is termed pH, so

$$pH = - \log \frac{K_1(CO_2)}{(HCO_3^-)}$$

But also, since log xy = log x + log y (from definition of logarithms)

$$pH = - \log K_1 - \log \frac{(CO_2)}{(HCO_3^-)}$$

In addition, since $\log \frac{x}{y} = - \log \frac{y}{x}$, the equation immediately above becomes:

$$(5)\ pH = - \log K_1 + \log \frac{(HCO_3^-)}{(CO_2)} = - \log K_1 + \log \frac{\text{bicarbonate}}{\text{carbon dioxide}}$$

One may note the similarity of the term pH to express - log (H^+), to the -log K_1 occurring in equation (5). Hence the expression pK_1 is quite logical and a convenient way to express the value - log K_1. We can therefore define the term:

$$pK_1 = - \log K_1$$

As long as such adjustments are being made, we can include in the pK_1 values employed, another factor to account for the "activity" of the materials involved which is not exactly the same as their concentration. We can thus define a pK' as follows:

$pK' = pK_1 + \log \theta$ where θ is the activity coefficient of the HCO_3^-.

This will make the equation just a little more exact. Employing this value, then, equation (5) becomes:

$$(6)\quad pH = pK' + \log \frac{(HCO_3^-)}{(CO_2)}$$

This is the "Henderson-Hasselbach equation" relating the carbon dioxide pressure, the bicarbonate ion concentration and the pH. Naturally, in order to use this equation, the value of pK' must be known. It has been carefully measured by Hastings and Sendroy (1925) who give a value at infinite dilution at 38°C of:

$$pK'_{\infty} = 6.33$$

In concentrations greater than infinite dilution the value will decrease slightly. At lower temperatures it will increase. The increase due to temperature is 0.005 units per degree (°C) drop in temperature.

This equation (6) has very wide use, but it is subject to certain limitations. It is well to point out its limitations before discussing it further. First, it neglects the second dissociation constant of carbonic acid (corresponding to the reaction $HCO_3^- = H^+ + CO_3^=$). However, E. J. Warburg (1922) has shown that if the pH is less than 8, the error arising from this neglect is unimportant. Second, the equation itself employs a term for bicarbonate concentration, (HCO_3^-), whose concentration one may not be able to determine exactly. For example, if $NaHCO_3$ is present, there will be HCO_3^- ion from the sodium bicarbonate and also bicarbonate ion from the dissociated carbonic acid (i.e., dissolved and dissociated CO_2). But again, E. J. Warburg (1922) showed that if the H^+ concentration was one one-hundredth (1/100) of the concentration of $NaHCO_3$ (or other metal bicarbonate), the bicarbonate concentration for use in the equation (6) could be taken as equivalent to that of the bicarbonate added. Neglect of the bicarbonate from carbonic acid, under these conditions, would cause an error of less than 1 part in 1000. Thus at pH 5 ($H^+ = 10^{-5}$M), the lowest bicarbonate concentration which could be employed would be 10^{-3}M (M/1000). At pH 7, the lowest bicarbonate concentration which could be employed would be 10^{-5} M (M/100,000). It is obvious that in the physiological range of pH 6-8 it would not be difficult to supply sufficient bicarbonate to overcome any error arising from the neglect of the second dissociation constant of carbonic acid.

In actual practice, using contemporary Warburg instruments (overall accuracy of 5%) it is found that if bicarbonate salts are employed, one can use the concentration of the bicarbonate supplied as the (HCO_3^-) in the equation, providing the bicarbonate concentration supplied is at least ten times that of the H^+ concentration. In addition, under most practical circumstances one may use the bicarbonate supplied as the total bicarbonate concentration providing it is at least ten times the concentration of other "carbon dioxide binding" materials.

The carbon dioxide concentration in the equation (6) is expressed in the same units as the bicarbonate, i.e., in moles per liter. Since the value usually known is the carbon dioxide pressure, the following equation (7) is used to convert pressure of carbon dioxide into moles per liter. Occasionally one will find equations (6) and (7) combined.

$$(7)\quad \text{carbon dioxide in moles per liter } (CO_2) = \frac{P\ \alpha\ pCO_2 \cdot 1000}{760 \cdot 22{,}400}$$

where P = atmospheric pressure
pCO_2 = pressure of CO_2 at the atmospheric pressure, P
α = solubility of CO_2

The term P/760 converts the atmospheric pressure to standard conditions, α represents the solubility in the solution involved, and 1000/22,400 is a factor to change α from liters/liter to moles/liter.

The "Henderson-Hasselbach equation" shows that in order to measure CO_2 at pH 7, there must be CO_2 in the atmosphere, since if CO_2 is 0, or

$$(6)\quad pH = pK' + \log (HCO_3^-)/(CO_2)$$

approaches 0, the factor $\log (HCO_3^-)/(CO_2)$ (equation (6)) becomes larger (hence the pH increases) or, if the pH is held low, the HCO_3^- becomes zero. At a pH of 5 or below, no bicarbonate or carbonate ion can exist, hence any CO_2 released will escape to the air. Thus one can measure CO_2 evolution from urea under the action of urease (which can occur at pH 5) without supplying CO_2 to the atmosphere. But most physiological reactions occur at pH 7, hence because of low levels of CO_2 in the air, either the bicarbonate concentration must be kept low, or CO_2 must be supplied in the air (if the pH is to be maintained at 7). It is obviously impossible to keep the bicarbonate low because the reaction of carbonic acid with tissue buffers tends to increase bicarbonate. The error arising from this source is only negligible in practice when the bicarbonate concentration is at least 10 times higher than that of tissue buffers. Hence the practical solution is to add CO_2 to the gas phase. It should be emphasized that if one fixes the bicarbonate concentration, one can obtain any pH between the ranges of 5 and 8 by adjusting the pCO_2 and *vice versa*. We shall work out several examples, but it is obvious that the situation can be altered to fit many other cases.

EXAMPLES OF THE USE OF THE HENDERSON-HASSELBACH EQUATION

Example 1: Suppose we wish to obtain a pH of 7 in M/1000 $NaHCO_3$ at 28°C., what concentration of CO_2 is necessary?

Equation (6) becomes:

$$7 = pK' + \log 10^{-3}/(CO_2)$$

pK' at 38°C. is 6.33. For each degree lower, 0.005 is subtracted from this value. Therefore at 28°C., pK' = 6.28

$$7 = 6.28 + \log 10^{-3}/CO_2 \quad \text{or}$$

$$\log 10^{-3}/CO_2 = 0.72 \quad \text{or}$$

$$\log 10^{-3} - \log CO_2 = 0.72$$

$$-3 - \log CO_2 = 0.72$$

$$\log CO_2 = -3.72 = 6.28 - 10$$

$$CO_2 = .0001906 \text{ moles/liter}$$

1.9×10^{-4} moles/liter of dissolved CO_2 are necessary to obtain a pH of 7 at a bicarbonate concentration of 10^{-3} molar. How much gas is required in the atmosphere to obtain 1.9×10^{-4} moles/liter in solution?

$$\text{moles/liter } CO_2 = (P \alpha pCO_2/760)\ (1000/22,400)$$

Suppose that the atmospheric pressure on the day the experiment was to be run was 740 mm. Hg, (i.e., P = 740) and α for CO_2 at 28° is 0.704. This is obtained from the α value of 0.759 at 25° and 0.665 at 30° by interpolation. Hence

$$1.9 \times 10^{-4} = (740 \times 0.704\ pCO_2/760)\ (1000/22,400)$$

$$pCO_2 \text{ (in atmospheres at 760 mm.)} = 1.9 \times 10^{-4} \times 760 \times 22.4/740 \times 0.704 =$$

$$.00622\ (\times 100 = 0.6\%\ CO_2; \text{ see on following page}).$$

Thus a pCO_2 of 0.00622 atmospheres (=$6.22 \times 10^{-3} \times 760 = 4.74$ mm. Hg or $4.74/760 \times 100 = 0.622\%$ CO_2) or 0.6% CO_2 in the air will give a pH of 7 at 740 mm. pressure at a bicarbonate concentration of 10^{-3} molar.

Example 2. Suppose that the same conditions hold except that the temperature is 38°C., and that the bicarbonate solution is 1.5×10^{-2} molar and the pH desired is 7.4 (this case is considered by Warburg, 1926).

$$7.4 = 6.33 + \log 1.5 \times 10^{-2}/CO_2$$

$$\log 1.5 \times 10^{-2} - \log CO_2 = 7.4 - 6.33 = 1.07$$

$$\log CO_2 = -(1.07 - \log 1.5 \times 10^{-2}) = -(1.07 - (8.17609 - 10))$$

$$= -(1.07 - (-1.82391)) = -2.89$$

CO_2 = antilog (-2.89 = antilog (7.11 - 10) = 1.288×10^{-3} $pCO_2 = 1.29 \times 10^{-3} \times$ 760 x 22.4/740 x 0.56 (α for CO_2 at 38° = 0.56) pCO_2 = .053 = 5.3%

Thus at 38°, at 740 mm. pressure, and 0.015 M bicarbonate, a gas mixture containing 5.3% CO_2 is necessary to obtain a pH of 7.4.

Example 3. In practice one usually finds it more convenient to employ a constant gas mixture and to vary the bicarbonate concentration. For bicarbonate concentrations in the range of M/100 bicarbonate, gas mixtures containing 5% CO_2 are employed; for M/1000 bicarbonate, gas mixtures containing 1% CO_2 are used. These are particularly convenient since the amount of carbonate occurring in solution under these conditions in negligible, hence the "Henderson-Hasselbach equation" holds.

Suppose one has an atmospheric pressure of 740 mm. Hg, 5% CO_2 in O_2, what concentration of bicarbonate is necessary to obtain a pH of 7? (28°).

$$CO_2 \text{ in moles/liter} = 740 \times (0.05) \times 0.704/760 \times 22.4 = 1.52 \times 10^{-3}$$

$$7 = 6.28 + \log NaHCO_3 - \log 1.52 \times 10^{-3} = 6.28 + \log NaHCO_3 + 2.818$$

$$\log NaHCO_3 = 7 - 9.1 = -2.10 = 7.9 - 10$$

$$NaHCO_3 = 8 \times 10^{-3} \text{ M.}$$

GRAPHICAL SIMPLIFICATION

One may note that the equation (6):

$$pH = pK' + \log HCO_3^-/CO_2$$

may be solved for $\log HCO_3^-$ and pH as follows:

$$pH = pK' + \log HCO_3^- - \log CO_2$$

$$-\log HCO_3 = pK' - \log CO_2 - pH$$

$$\log HCO_3 = +pH - (pK' - \log CO_2)$$

From this one can readily graph the relationships at various pH's and at various temperatures since this relationship is a straight line function. This has been done for 25, 28, 30 and 37°C. in the accompanying graph (Fig. 7). Thus for any desired pH in a gas atmosphere containing 5% CO_2, one need only read from the graph the bicarbonate concentration required. This graph is constructed for an atmospheric pressure of 740 mm. Hg, but the method of preparation of this graph can be applied to any other pressure, to any other CO_2 concentration, to any other temperature, etc. It is suggested that a series of these graphs be prepared to cover the conditions under which one is working. Certain simplifications can be introduced in the calculations after one has a familiarity with the process, but to describe them at this point would probably introduce confusion.

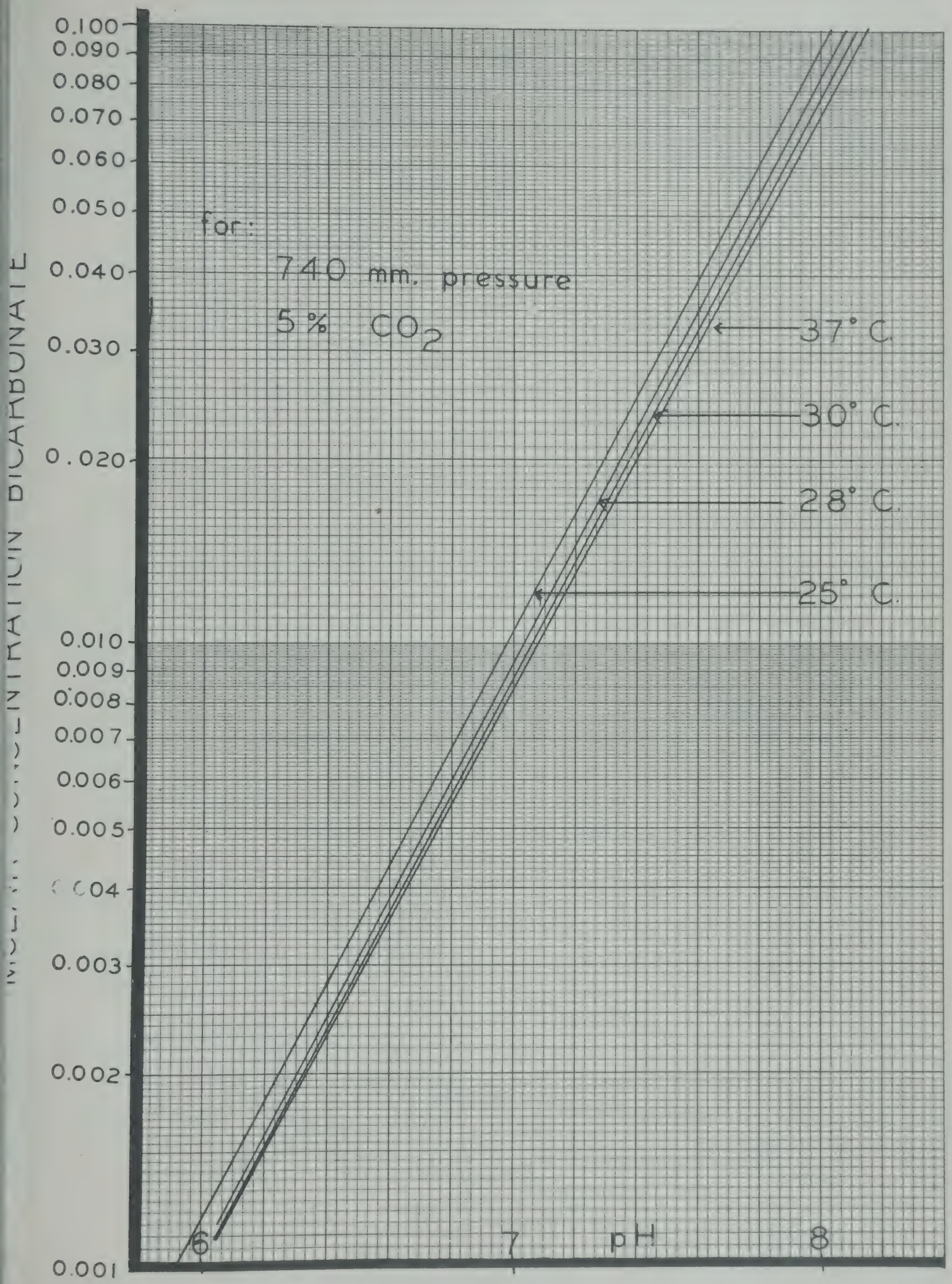

Fig. 7. Relationships between bicarbonate concentration and pH.

THE USE OF BICARBONATE BUFFERS FOR MEASURING ACID PRODUCTION UNDER ANAEROBIC CONDITIONS

If one removes the oxygen, there can, of course, be no respiration (i.e., oxygen uptake). If one employs a bicarbonate buffer in an atmosphere of CO_2, as described in the previous section, CO_2 released by the cells will escape into the atmosphere and thus can be measured manometrically. Procedures for obtaining anaerobic conditions are described in Chapter 5. Furthermore, if any acid should be produced under these conditions it would combine with metallic ions formerly associated with bicarbonate, thus CO_2 would be released into the atmosphere. Hence, one can measure both acid production and CO_2 liberation not due to acid by means of bicarbonate buffers under anaerobic conditions.

If one employs a tissue which does not liberate CO_2 when metabolizing anaerobically, it is simple to determine the acid produced since one can take the CO_2 released from bicarbonate as a measure of the acid production. Another way which has been used to differentiate the "fermentation" CO_2 from acid, is to determine the CO_2 production in the absence of bicarbonate and to subtract this from the CO_2 produced in its presence. However, the determination of the rate of CO_2 production in the absence of bicarbonate is difficult with most tissues, since to avoid any great effect of bicarbonate produced by interaction with buffers ("CO_2 retention") one must work in low concentrations of buffers. The CO_2 and acid liberated soon reduce the pH from the initial point and frequently stop respiration.

A method which permits one to differentiate between the "metabolic CO_2" and acid (both being measured as CO_2 liberated under anaerobic conditions) is the following:

Any CO_2 liberation as such will not influence the bicarbonate concentration , but any acid which is liberated will produce CO_2 from bicarbonate, i.e., will decrease the amount of bicarbonate. Hence by adding sufficient acid to measure the bicarbonate remaining one can estimate the acid and the metabolic CO_2. Two manometers (plus a thermobarometer) are required. Each has the tissue, buffer, bicarbonate and is in equilibrium with a known pressure of CO_2. Each has acid (usually 0.1 - 0.5 ml. 3N H_2SO_4) in the side arm or in "Keilin tubes" (see Chapter 5). The acid is sufficient to instantly stop the metabolism and to bring the pH to below 5 (usually to between pH 1 and 2) upon addition. In some rare cases the tissues are resistant to acid so a poison is added along with the acid. After equilibration the acid is tipped in to one of the manometers. This gives the total (initial) bicarbonate (as CO_2) available. CO_2 output is measured for the experimental period in the other flask. Acid is then tipped in. The amount of CO_2 released by the acid measures the residual bicarbonate. The difference between this and the initial bicarbonate gives the amount of CO_2 produced by acid formation. Any other CO_2 produced is that produced by the tissue as CO_2 not as acid (Warburg, 1914).

The usual Warburg instrument is capable of measuring 300 µl. of CO_2 with ease. Since 1 ml. of 0.001 molar $NaHCO_3$ will release 22.4 µl. of CO_2, one may use as much as 1 ml. of 0.01 M $NaHCO_3$ (which at 37° at 5% CO_2 gives a pH of 7.08) and release all the CO_2 as gas by tipping an acid without extending the fluid in the manometer beyond its graduated range providing one starts the experiment with the open end of the manometer at a low level (less than 10 cm.).

Such measurement of acid production under anaerobic conditions is usually spoken of as "glycolysis" and, when animal tissues are employed, the acid is largely lactic acid. In other cases, however, one cannot make the assumption that the product is lactic acid or that it is largely lactic acid. Hence, calculation of the acid produced as lactic acid is likely to result in error. Fortunately there is now available a very specific chemical method for the determination of lactic acid in the flasks (see Chapter 10), so that one can actually determine how much of the acid produced was lactic acid.

THE INFLUENCE OF CARBONATE

It has been pointed out that the "Henderson-Hasselbach" equation neglects the second dissociation constant of carbonic acid. In the presence of bicarbonate and CO_2, most of the Na^+ and K^+ ions are associated with bicarbonate but some carbonate does always exist. The question is, is there enough of this to make any difference? The production of CO_2 or of acid will tend to combine with the carbonate converting it to bicarbonate and this amount will escape manometric estimation since no gas is released. The equations involved are:

$$(H^+)\ (HCO_3^-)/H_2CO_3 = (H^+)\ (HCO_3^-)/(CO_2) = K_1 = 3 \times 10^{-7}.$$

$$(H^+)\ (CO_3^{--})/HCO_3^- = K_2 = 6 \times 10^{-11}$$

$$K_1/K_2 = K' = \frac{(H^+)\ (HCO_3^-)}{CO_2} \Big/ \frac{(H^+)\ (CO_3^=)}{HCO_3^-} = (HCO_3^-)^2/(CO_2)\ (CO_3^{--})$$

Thus $K' = K_1/K_2 = 3 \times 10^{-7}/6 \times 10^{-11} = 0.5 \times 10^{+4} = 5 \times 10^{+3} = 5000$

Using 5% CO_2 with a bicarbonate concentration of 0.01 molar at 25°C., the concentration of carbonate is:

$$CO_3^{--} = (H_2CO_3^-)^2/CO_2K' = (0.01)^2/1.52 \times 10^{-3} \times 5 \times 10^{-3} = 10^{-4}/5 \times 1.52 =$$

$$1.32 \times 10^{-5} \text{ moles } CO_3^{--}/\text{liter}$$

In short, a negligible quantity.

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Chapter Four

THE "INDIRECT" METHOD OF WARBURG

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Chapter Four

THE "INDIRECT" METHOD OF WARBURG

The one basic difficulty with the "direct" manometric methods described in Chapters 1 and 2, was that, in order to measure oxygen uptake, one was forced to work in an atmosphere free from CO_2. Yet in many tissues there was always the suspicion that perhaps such measurements were not valid for this very reason, i.e., the rate of respiration, and even the course of the reactions, might be altered in the absence of CO_2. For example, decarboxylation reactions, which in the body might be in equilibrium with CO_2, would be forced to completion if the CO_2 were all removed, and the influence of this situation upon the rate or course of oxygen uptake could not be estimated. There was no way of determining whether or not the presence or absence of CO_2 had an effect.

This problem was solved, at least partially, by Warburg (1924, 1926) who described a method which has since been termed Warburg's "Indirect Method". The principle upon which the method is based is that if one has two gases of markedly different solubility, one can measure the exchange in both by comparing the manometric changes when exactly the same reaction is carried out in two flasks which have either markedly different fluid volumes, or markedly different gas volumes.

PRINCIPLES AND DERIVATIONS

We shall confine ourselves to the discussion of the case in which one has two flasks of approximately equal volume containing markedly different volumes of fluid. It will be apparent that exactly the same equations hold for other circumstances (see later). We shall also confine the discussion to the gases O_2 and CO_2 (whose solubilities are markedly different) but it will be also apparent that any other gases may be substituted providing their solubility in the fluids in the flasks is different.

Suppose that we have two flasks of approximately the same volume, but containing different volumes of fluid. The same amount of tissue is placed in each and the same reaction occurs in each. In order to distinguish the two flasks, we shall use small letter symbols for the flask containing less fluid. (h = change in reading on manometer, k_{O_2} = flask constant for O_2, k_{CO_2} flask constant for CO_2, $-x_{O_2}$ = amount of O_2 taken up, $+x_{CO_2}$ = amount of CO_2 liberated, etc.) and capital letters (H, K_{O_2}, K_{CO_2}, X_{O_2}, X_{CO_2} etc.) for the flask containing the larger amount of fluid. This is purely conventional and it is obvious that the symbols can be exchanged without influencing the derivation in the least. We shall also employ a different derivation than was used by Warburg (1924) because, to our minds, the derivation below is easier to understand and serves to emphasize that the derivation employed by Warburg is perfectly general; in short, that it is applicable to situations other than those described by Warburg.

Taking first the flask with the smaller volume of fluid, the change observed on the manometer (h) is due to two things:

(a) The uptake of oxygen (h_{O_2})

(b) The release of CO_2 (h_{CO_2})

Thus:

$$h_{(observed)} = h_{O_2} + h_{CO_2}$$

but by definition:

$$h_{O_2} = x_{O_2}/k_{O_2} \text{ and } h_{CO_2} = x_{CO_2}/k_{CO_2} \text{ (since } x_{O_2} = h_{O_2}k_{O_2})$$

Therefore:

$$(8)\quad h_{(observed)} = x_{O_2}/k_{O_2} + x_{CO_2}/k_{CO_2}$$

If the same reaction has occurred in the second flask containing the larger volume of fluid, the change observed on the manometer (H) will similarly be:

$$(9)\quad H = X_{O_2}/K_{O_2} + X_{CO_2}/K_{CO_2}$$

However if the identical reaction has occurred in each flask, the amount of oxygen taken up (x_{O_2}, X_{O_2}) in each case should be the same, and the amount of CO_2 released should be the same (X_{CO_2}, x_{CO_2}) hence:

$$(9a)\quad x_{O_2} = X_{O_2};\ \text{and}\ x_{CO_2} = X_{CO_2}$$

Thus one may substitute in either equation (8) or (9) yielding:

$$(10)\ \text{from equation (8)}\quad h = X_{O_2}/k_{O_2} + X_{CO_2}/k_{CO_2} \qquad \text{or}$$

$$(11)\ \text{from equation (9)}\quad = x_{O_2}/K_{O_2} + X_{CO_2}/K_{CO_2}$$

DETERMINATION OF OXYGEN EXCHANGE

One can combine equations (8) and (9) in another way. Suppose that one solves equation (8) for x_{CO_2} in the following way:

$$\text{equation (8)}\quad h = x_{O_2}/k_{O_2} + x_{CO_2}/k_{CO_2}\ \text{hence,}$$

$$x_{CO_2}/k_{CO_2} = h - x_{O_2}/k_{O_2}\ \text{or}$$

$$(12)\quad x_{CO_2} = k_{CO_2}\,(h - x_{O_2}/k_{O_2})$$

From equation (9), in the same way one obtains

$$(13)\quad X_{CO_2} = K_{CO_2}\,(H - X_{O_2}/K_{O_2})$$

But as was shown in equation (9a) $x_{CO_2} = X_{CO_2}$, hence equations (12) and (13) are equal to one another, or

$$(14)\quad k_{CO_2}\,(h - x_{O_2}/k_{O_2}) = K_{CO_2}\,(H - X_{O_2}/K_{O_2})$$

But equation (9a) also showed that $X_{O_2} = x_{O_2}$, hence this can be substituted to yield equation (15):

$$(15)\quad k_{CO_2}(h - X_{O_2}/k_{O_2}) = K_{CO_2}(H - X_{O_2}/K_{O_2})$$

The thing we are interested in determining is X_{O_2} (or x_{O_2}, since both are equal), hence solving for X_{O_2} as follows:

Multiply out equation (15):

$$hk_{CO_2} - X_{O_2}k_{CO_2}/k_{O_2} = HK_{CO_2} - X_{O_2}K_{CO_2}/K_{O_2}$$

Transpose terms:

$$X_{O_2}K_{CO_2}/K_{O_2} - X_{O_2}k_{CO_2}/k_{O_2} = HK_{CO_2} - hk_{CO_2}$$

Take out X_{O_2}:

$$X_{O_2}(K_{CO_2}/K_{O_2} - k_{CO_2}/k_{O_2}) = HK_{CO_2} - hk_{CO_2}$$

$$(16)\quad x_{O_2} = X_{O_2} = \frac{HK_{CO_2} - hk_{CO_2}}{\frac{K_{CO_2}}{K_{O_2}} - \frac{k_{CO_2}}{k_{O_2}}}$$

Note: One will sometimes find this equation written with the capital and small letters interchanged (Dixon, 1943). It may be noted that this is exactly the same equation except that the sign of both numerator and denominator have been changed.

The oxygen taken up (x_{O_2} or X_{O_2}) depends on the reading of one flask (H) times its CO_2 constant (K_{CO_2}) less the reading of the other flask (h) times its CO_2 constant (k_{CO_2}) divided by a constant which is calculated from the ratio of the CO_2 and oxygen constants of the first flask less the ratio of the same constants for the second flask. Equation (16) may be modified to:

$$(17)\quad x_{O_2} = X_{O_2} = HK_{CO_2} - hk_{CO_2}/C_{(O_2)}$$

where $C_{(O_2)}$ = constant = $K_{CO_2}/K_{O_2} - k_{CO_2}/k_{O_2}$

Once this constant ($C_{(O_2)}$) has been calculated from given experimental conditions, the calculation of the oxygen uptake becomes relatively simple. Normally one prepares a table of this sort:

(1)	(2)	(3)	(4)	(5)	(6)
H	HK_{CO_2}	h	hk_{CO_2}	Difference HK_{CO_2}-hk_{CO_2}	$\mu l.O_2$
Observed, corrected for thermobarometer changes.	Multiply column (1) by its flask constant for CO_2.	Observed, corrected for thermobarometer changes.	Multiply column (3) by its flask constant for CO_2.	Subtract column (4) from column (2).	Divide column (5) by $C_{(O_2)}$.

In making such calculations one must be careful to retain the algebraic sign of each measurement, i.e., if the level of fluid in the manometer drops, -h is used. If it rises, +h. (See example below).

DETERMINATION OF CO_2 EXCHANGE

In an entirely similar manner one may use equations (8) and (9) to calculate the CO_2 exchange. Suppose that one solves equation (8) for x_{O_2} in the following way:

From equation (8) $h = x_{O_2}/h_{O_2} + xCO_2/k_{CO_2}$ hence

$$(18)\quad x_{O_2} = (h - x_{CO_2}/k_{CO_2})k_{O_2}$$

From equation (9) in the same way one obtains

$$(19)\quad X_{O_2} = (H - X_{CO_2}/K_{CO_2})K_{O_2}$$

But $x_{O_2} = X_{O_2}$, from equation (9a), hence equations (18) and (19) are equal to one another, or:

$$(20)\quad (h - x_{CO_2}/k_{CO_2})k_{O_2} = (H - XCO_2/KCO_2)K_{O_2}$$

Equation (9a) also showed that $x_{CO_2} = X_{CO_2}$; hence for x_{CO_2} we can substitute X_{CO_2}.

$$(21)\quad (h - X_{CO_2}/k_{CO_2})k_{O_2} = (H - X_{CO_2}/K_{CO_2})K_{O_2}$$

Equation (21) may be solved for X_{CO_2} (the CO_2 liberation or uptake which we are trying to determine) as follows:

Multiply out (21): $hk_{O_2} - X_{CO_2}k_{O_2}/k_{CO_2} = HK_{O_2} - X_{CO_2}K_{O_2}/K_{CO_2}$

Transpose terms: $X_{CO_2}K_{O_2}/K_{CO_2} - X_{CO_2}k_{O_2}/k_{CO_2} = HK_{O_2} - hk_{O_2}$

Take out X_{CO_2}: $X_{CO_2}(K_{O_2}/K_{CO_2} - k_{O_2}/k_{CO_2}) = HK_{O_2} - hk_{O_2}$ hence,

$$(22)\quad x_{CO_2} = X_{CO_2} = (HK_{O_2} - hk_{O_2})/\left(\frac{K_{O_2}}{K_{CO_2}} - \frac{k_{O_2}}{k_{CO_2}}\right) = (HK_{O_2} - hk_{O_2})/C_{CO_2}$$

The carbon dioxide taken up (if X_{CO_2} = -) or given off (if X_{CO_2} = +) is equal to the change in reading on one flask times the oxygen constant of that flask, less the change of reading in the other flask times its oxygen constant, divided by a constant.

Once this constant:

$$C_{(CO_2)} = K_{O_2}/K_{CO_2} - k_{O_2}/k_{CO_2}$$

has been calculated from given experimental conditions, the calculation of the CO_2 exchange becomes relatively simple. A table such as that used for oxygen may be used:

(1)	(2)	(3)	(4)	(5)	(6)
H	HK_{O_2}	h	hk_{O_2}	Difference HK_{O_2}-hk_{O_2}	$\mu l.CO_2$
Observed, corrected for thermobarometer changes.	Multiply column (1) by its flask constant for O_2.	Observed corrected for thermobarometer changes.	Multiply column (3) by its flask constant for O_2.	Subtract column (4) from column (2).	Divide column (5) by constant (C_{CO_2}). If value in column is + CO_2 is liberated; if - CO_2 taken up.

SUMMARY

By using two flasks of approximately equal volume, one of which contains more fluid than the other, two equations were derived in the previous paragraphs which permit one to determine the oxygen uptake (or release) and the CO_2 production (or uptake). These equations were:

$$(17)\quad x_{O_2} = X_{O_2} = HK_{CO_2} - hk_{CO_2}/C_{(O_2)}$$

$$(22)\quad x_{CO_2} = X_{CO_2} = HK_{O_2} - hk_{O_2}/C_{CO_2}$$

Where the $C_{(O_2)}$ and C_{CO_2} represent constants calculable from the experimental set up employed.

This procedure permits one to determine O_2 uptake and CO_2 production in the presence of adequate supplies of CO_2, and thus enables one, by a comparison with the results of the "Direct Methods" described in Chapters 1 and 2, to determine whether or not CO_2 does influence the rate or the course of the process involved.

THE EQUATION CONSTANTS

The constants employed in the equations (No. (17), (22)) for calculating the oxygen and carbon dioxide exchange were defined as follows:

$$C_{(O_2)} = K_{CO_2}/K_{O_2} - k_{CO_2}/k_{O_2}$$

$$C_{CO_2} = K_{O_2}/K_{CO_2} - k_{O_2}/k_{CO_2}$$

In Chapter 6 methods of determining and calculating the flask constants (k or K) are described. Here it may be noted that:

$$K_{CO_2}/K_{O_2} = 1/\frac{K_{O_2}}{K_{CO_2}}$$

It is therefore convenient to have not only the oxygen and carbon dioxide constants of each flask recorded for various volumes, but also their ratio. (It is not true, however, that C_{CO_2} is the reciprocal of $C_{(O_2)}$.

EXAMPLE OF THE USE OF WARBURG'S INDIRECT METHOD

We may cite an experiment on algae. 1 ml. of a suspension of Chlorella cells (containing 0.1 ml. packed wet cells) was placed in each of two Warburg flasks (No. 5 total volume = 13.9 ml.; No. 7, total volume 13.3 ml.). In the first flask was placed 1 ml. of M/1000 phosphate buffer (pH 4.5) (flask No. 5, $k_{O_2} = 1.08$, $k_{CO_2} = 1.22$); in the second, 5 ml. of the same phosphate solution was added (flask No. 7, $K_{O_2} = 0.67$, $K_{CO_2} = 1.09$). No added CO_2 was supplied to the air. After equilibration at 28°, readings were taken at 5 minute intervals. The readings obtained (corrected for thermobarometer changes) were as follows:

	Flask no. 5(h)	Flask no. 7 (H)
First 5 minutes	0	-4.5
Second 5 minutes	-2	-5.5
Third 5 minutes	-1	-7.0
Fourth 5 minutes	0	-3.5

Oxygen uptake calculations are shown in Table IX, CO_2 exchange reactions are shown in Table X.

TABLE IX

Oxygen Exchange

	Flask 7		Flask 5				
Time	H(mm)	HK_{CO_2}	h(mm)	hk_{CO_2}	Difference	$\mu l.O_2$*	Sum
5	-4.5	-4.9	0	0	-4.9	-9.8*	-9.8
10	-5.5	-6.0	-2	-2.4	-3.6	-7.2	-17.0
15	-7.0	-7.6	-1	-1.2	-6.4	-12.8	-29.8
20	-3.5	-3.8	0	0	-3.8	-7.6	-37.4

$K_{CO_2} = 1.09$ $k_{CO_2} = 1.22$

* $= -4.9/C_{(O_2)} = -4.9/.5 = -9.8$

$K_{CO_2}/K_{O_2} = 1.09/0.67 = 1.63$ $\quad k_{CO_2}/k_{O_2} = 1.22/1.08 = 1.13$

$C_{(O_2)} = K_{CO_2}/K_{O_2} - k_{CO_2}/k_{O_2} = 1.63 - 1.13 = 0.50$

TABLE X

Carbon Dioxide Exchange

	Flask 7		Flask 5				
Time	H	HK_{O_2}	h	hk_{O_2}	Difference	$\mu l.CO_2$	Sum
5	-4.5	-3.0	0	0	-3.0	+11.1*	+11.1
10	-5.5	-3.7	-2	-2.2	-1.5	+ 5.6	+16.7
15	-7.0	-4.7	-1	-1.1	-3.6	+13.3	+30.0
20	-3.5	-2.3	0	0	-2.3	+ 8.5	+38.5

$K_{O_2} = 0.67$ $\qquad k_{O_2} = 1.08$

$C_{CO_2} = K_{O_2}/K_{CO_2} - k_{O_2}/k_{CO_2} = 1/1.63 - 1/1.13 = 0.615 - 0.885 = -0.27$ (see Table X)

* i.e. -3.0/-0.27 = +11.1

The data thus obtained have been plotted in Fig. 8 from which it is apparent that the R. Q. is very close to 1, i.e., for every molecule of oxygen consumed, one molecule of CO_2 is liberated. The example has been chosen to show that even relatively small changes in volume of either gas can be estimated quite accurately. It has also been chosen to illustrate that, under the conditions employed (i.e., pH 4.5) it was not necessary to add CO_2 to the air, in order to measure the respiration. In Fig. 8 we have also drawn in the oxygen uptake curve obtained on the same algae when the CO_2 was absorbed by KOH (in the Warburg "Direct" Method (Chapter 1)). Comparison of the two curves shows that the presence of CO_2 does have an effect in this case, since respiration in the presence of CO_2 is less than respiration in its absence.

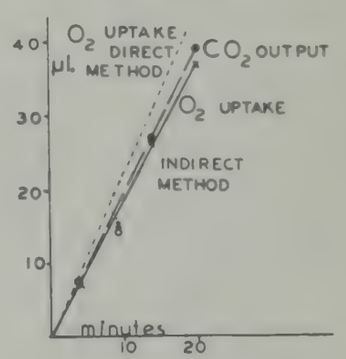

Fig. 8. Example of the use of the Warburg Indirect Method. See text.

APPLICATION TO OTHER GASES

It should be pointed out that this type of system can be applied to gases other than CO_2 and O_2. For gases "a" and "b", the equations (no. (17), (22)) become

$$x_a = X_a = \frac{HK_b - hk_b}{\frac{K_b}{K_a} - \frac{k_b}{k_a}} \quad \text{and} \quad x_b = X_b = \frac{HK_a - hk_a}{\frac{K_a}{K_b} - \frac{k_a}{K_b}}$$

One may note that should gases "a" and "b" have the same solubility, then $K_a = K_b$ and $k_a = k_b$ hence both equations become zero. Hence one can only apply this method to gases whose solubility differs appreciably.

PRACTICAL USE OF INDIRECT METHODS

In order to obtain a result which one can calculate, one can do any of three things:

1. Using flasks of approximately the same volume, employ markedly different volumes of fluid.
2. Using the same volume of fluid, employ flasks of different volume. (See, for example, Emerson and Lewis, 1941 and Fig. 18, page 56.)

3. Using flasks of the same volume, employ differing amounts of fluid, and markedly different amounts of tissue such that the ratio of tissue to fluid medium is the same in both cases. The derivations of this third type of use are somewhat different than previously described. Since we have not used this procedure, and since it offers no advantages over the two procedures listed above, we omit consideration of it here. Essentially one holds the tissue to fluid medium ratio constant, divides the observed manometer changes by the amount of tissue, and then calculates as described in the examples given. We have yet to convince ourselves that this is a justifiable procedure, but Dixon (1943, page 77) considers it "preferable" to procedure 2 (above).

For a given situation, not all of these are equally suitable. Suppose, for example, that one had a reaction whose rate was dependent on the concentration of a diffusible substance (say a coenzyme present in the tissue). When suspended in a large volume of fluid (as in the case of one of the samples in procedure 1) this essential substance may diffuse out and thus alter the rates of reaction such that the two flasks will not carry out the same reaction at the same speed. Hence, procedure 1 would give erroneous results. It is evident, however, that procedure 2 would work. Other circumstances would make procedure 1 better than 2, etc.

The derivations we have described above are somewhat different from those given by Warburg (1924). They have been developed in this way to emphasize the broad applicability of this method. Warburg's treatment, while a perfectly general one, has been more or less interpreted as a specific solution to the problem, and workers using the method have usually attempted to duplicate the conditions used by Warburg, rather than to employ the principles he emphasized in methods more suited to their conditions. It is not necessary for the success of this method to use the modified flasks Warburg describes, to work in bicarbonate buffers at the concentration and at the pH that he employs, nor is it necessary to work in an atmosphere of 5% CO_2. These conditions may be altered by the investigator at will and by the application of the laws of CO_2 - bicarbonate - pH equilibria, outlined in Chapter 3, a wide range of conditions may be employed.

ADAPTATION TO MORE COMPLEX MEASUREMENTS

In the previous derivations and discussion we have described how it is possible to determine two gases simultaneously if their solubilities differ. One would have a very reasonable chance of determining oxygen and hydrogen, for example. Physiologically, however, we are interested mostly in oxygen and carbon dioxide, and, as discussed in Chapter 3, carbon dioxide while obeying the general laws of solubility, also forms carbonic acid which can react with bases, and this alters the entire "solubility" picture.

If one is studying a reaction which produces no acid, but only CO_2, and can operate at a pH below 5 (for example, urease acting on urea) one may employ a system with no CO_2 added to the atmosphere. Such reactions are, however, quite rare. Examples of oxygen uptake and CO_2 release under these circumstances would be the respiration of acid tolerant sulfur bacteria (Vogler, LePage and Umbreit, 1941), but with few exceptions (Vogler, 1942) such reactions are not sensitive to the presence of CO_2 and can thus be more readily measured by more direct methods.

In the more normal physiological ranges, bicarbonate buffers may be employed in which case a CO_2 pressure in the atmosphere is required (see Chapter 3) in order to maintain a given pH. If the concentration of bicarbonate ion is at least 10 times greater than any other carbon dioxide retaining agent, the practical error involved from CO_2 retention by other materials is usually negligible. Thus it is possible to use a Ringer's solution with bicarbonate at a definite CO_2 pressure and obtain adequate measurements of gas exchange. It is under these circumstances that the method has been most widely used. As discussed previously, another difficulty arises if acid is produced as well as CO_2, since both will appear in the pressure changes as CO_2. What has usually been done is to assume a definite and constant R. Q. (usually taken as 0.9 or 1.0), i.e., for every oxygen taken up, one (or 0.9) CO_2 is released. One then takes any extra CO_2 beyond this figure as being due to acid production. But frequently, far from permitting assumptions of a definite and constant R. Q., this has been just the point which one wished to measure. Actually the problem of distinguishing between "respiratory CO_2" and acid production under these circumstances has never been critically solved although ingenious methods which approach a solution have been devised (Dixon, 1943).

If one wishes to work in media containing large amounts of CO_2-binding materials (protein, serum, etc.), the retention of CO_2 in the medium may become so large as to entirely invalidate the results of such measurements. The problem of "serum retention" (Warburg, 1925) has been approached and a reasonably satisfactory solution supplied for a few cases. But so complex do the conditions of operation become that most workers have studiously avoided such experiments. It is beyond the scope of this manual to describe these methods since they are admittedly too advanced for the beginner and are, in addition, of rather limited application. Before undertaking such studies a relatively long experience with manometric methods is probably necessary. Descriptions of the techniques employed and the theory upon which they are based will be found in papers by Warburg (1925), Warburg (1926), Warburg, Kubowitz, and Christian (1931), Dixon (1943), Dixon and Elliott (1930). The most general use of the "indirect" methods of Warburg have been in the determination of whether the presence of CO_2 (as bicarbonate) does indeed influence the reactions one is studying. If CO_2 is without effect, the "direct" methods are convenient and generally preferable. A short discussion of "retention" will be found in Chapter 11.

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Chapter Five

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Chapter Five

USEFUL TECHNIQUES FOR USE WITH MANOMETRIC METHODS

The practical operation of any instrument always involves details which are not readily recognized by the description of the procedures or the theory of the instrument. We have collected below some of the techniques which are useful when one is using the Warburg instrument.

CLEANING GLASSWARE

All glassware must be carefully cleaned. Four general methods are in use for cleaning flasks:

1. Dichromate method:
   (a) Place flasks in gasoline to remove grease.
   (b) Wash with water.
   (c) Place in cleaning solution for 12 - 24 hours. (Cleaning solution: dissolve 63 g. sodium (or potassium) dichromate by heating with 35 ml. water. Add conc. H_2SO_4 to 1 liter).
   (d) Remove, wash by rinsing in distilled water at least six times preferably under a flowing stream.
2. Permanganate method:
   (a) Place in gasoline for 30 minutes to remove grease. Wash in water.
   (b) Transfer to permanganate solution for 2-3 hours. (Permanganate solution: 20 g. $KMnO_4$, 50 g. NaOH to 1 liter water).
   (c) Transfer to a saturated solution of oxalic acid.
   (d) Wash several times in hot distilled water.
3. Nitric acid method:
   (a) Place in gasoline for 30 minutes to remove grease.
   (b) Wash with water.
   (c) Transfer to a mixture of equal parts of concentrated H_2SO_4 and HNO_3. Frequently the flasks are boiled in this mixture.
   (d) After several hours, remove, wash several times in distilled water.
4. Calgon method:
   (a) Remove grease with gasoline and cotton swab. Rinse with water.
   (b) Immerse in a pan containing 1 teaspoon Calgon per 2 quarts of water. Boil gently for 30 minutes.
   (c) Rinse several times with distilled water.

OPERATIONAL TECHNIQUES

Grease: The grease used in lubricating the ground glass joints of the flask-manometer connections or the plugs for the side arms is usually either anhydrous lanolin or heavy vaseline. The grease used for the stopcock on the manometer is preferably a good stopcock grease such as is used for burettes.

At high temperatures Celevacene light (Distillation Products Co.) is useful because of its small change in consistency with changing temperature.

Brodie's Solution: A formula of a convenient solution is the following:

23 grams NaCl
5 grams Sodium choleate (Merck)
in 500 ml. water
Density 1.033
P_o = 10000

Evan Blue (200 mg./liter) was found by Bain to be an excellent dye for the fluid; other dyes may be used, but some of these tend to decompose in the manometer. Obviously many materials may be used in the manometers, and for each it is only necessary to know its density.

Clerici Solution:

7 grams thallium formate
7 grams thallium malonate
1 ml. water
Density about 4; P_o value about 2500

Mercury: It is convenient, when using mercury, to place a drop of water at the top of each column. This permits the mercury to flow freely. Density about 13.6 (dependent on temperature); P_o = 760.

Removing bubbles in manometer column: If the column of Brodie's fluid is broken in the manometer it may be readily joined again by rapidly depressing the rubber Brodie fluid reservoir with the finger and then releasing the pressure slowly. Repeating this soon raises the bubbles to the surface of the liquid.

READING THE MANOMETER AFTER IT HAS PASSED THE GRADUATED RANGE

W. W. Umbreit

It sometimes happens that, due to the addition of gases or their absorption, the adjustment of the fluid in the closed arm of the manometer results in the fluid in the open arm coming to rest at a point off the graduated scale of the open arm. A method to adjust the zero point on the closed arm so that a reading can be obtained is the following (Vogler, 1942):

Adjust the fluid in the closed arm until that in the open arm is on the scale. The distance one has moved the fluid in the closed arm from the zero point is called "e". Record the reading in the open arm. Adjust the closed arm so that the fluid is a distance 2e from the zero point. Record the reading in the open arm. The difference between the two readings of the open arm is the amount to be added to or subtracted from the first reading to give the actual reading if the closed arm had been at the zero point.

Theoretical: If the volume of gas (V_g) be decreased x by raising the level of manometer fluid in the closed arm by e cm., the corresponding pressure P_o will be increased by y so that:

$$(V_g - x)(P_o + y) = V_g P_o \quad \text{or}$$

$$V_g\, y = x\,(y + P_o) \quad \text{(See Fig. 9).}$$

Similarly if the fluid in the closed arm be raised f cm., the corresponding volume change (x') will cause an increased y' so that:

$$V_g\, y' = x'(y' + P_o)$$

Thus:

$$y/y' = x\,(y + P_o)/x'(y' + P_o)$$

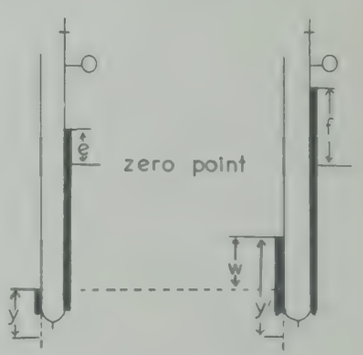

Fig. 9. Diagram illustrating the method of reading the manometer after it has passed the graduated range.

Since $x = \pi r^2 e$ and $x' = \pi r^2 f$, where "r" is radius of the capillary tube of the manometer:

$$y/y' = \pi r^2 e\,(y + P_o)/\pi r^2 f\,(y' + P_o) = e\,(y + P_o)/f\,(y' + P_o)$$

Since P_o is large (10,000 mm. of Brodie's solution), and y and y' are small, an accuracy of 1% is possible, if y and y' are not greater than 100 mm. (10 cm.), by considering $y + P_o = y' + P_o$, from which $y/y' = e/f$.

If W were defined as the difference between the reading at y and y' (closed arm at e or f), then

$$y' - y = W \quad \text{or} \quad y' = W + y$$

Thus:

$$y = \frac{y'e}{f} = \frac{(W + y)\, e}{f} = \frac{We + ye}{f}$$

$$fy = We + ye$$

$$y\,(f - e) = We$$

$$y = \frac{We}{f-e}$$

If f were chosen to equal 2e, then y = W.

"y" is the distance off the scale (when closed arm is at zero point) from the first reading on the scale (when closed arm is at e).

ILLUSTRATION 1. Oxygen uptake is unexpectedly rapid and upon returning the closed arm to its zero point (250 mm.) the open end does not reach zero. By adjusting the closed arm to 260; the reading is 3; by adjusting to 270, the reading is 8. Hence e = 10; f = 20; W = 5; y = We/f - e = 5. Reading at zero point was 3 - 5 = -2.

ILLUSTRATION 2. In order to obtain a reading on the scale, it was necessary to adjust the closed arm to 280; (e = 30 from zero point of 250) hence 2e is impossible (310), since closed arm scale will not read to this point. Reading at 280 = 5. Reading at 300 = 16.

$$y = We/f - e = 11 \times 30/50 - 30 = 330/20 = 16.5$$

Reading at zero point = 5 - 16.5 = -11.5.

ANAEROBIC MEASUREMENTS

R. H. Burris

Anaerobic conditions are generally obtained in respiration vessels by thorough flushing with nitrogen freed of oxygen. Tank nitrogen is passed over heated copper turnings in a combustion tube and thence through a manifold to the Warburg flasks which are shaking in the bath. Ten minutes of slow flushing should suffice to remove oxygen. If some gas other than nitrogen, e.g., hydrogen or helium, is desired in the vessels the same procedure may be followed (with hydrogen the combustion train must be flushed to remove air before the heat is applied).

The evacuation procedure described under "Altering Gas Mixtures" may be used to obtain anaerobic conditions. Using four evacuations to 75 mm. Hg residual pressure should leave only about 0.3 µl. of the original oxygen in a 15 ml. flask. The gas may be added directly from a heated copper packed combustion tube.

Alkaline pyrogallol in an absorption tower is less satisfactory than hot copper for removing contaminating oxygen. Small pieces of freshly cut yellow phosphorus added to the center well or side-bulb of a Warburg flask will remove residual oxygen that may remain after incomplete flushing. However, a thorough flushing with oxygen-free gas should obviate the need for this. The addition of phosphorus is somewhat undesirable, as the P_2O_5 fumes which form before oxygen-free gas is passed through the system may be absorbed with resultant alteration in the pH of the medium. When phosphorus is depended on to rid the flask of oxygen, initiation of the reaction must be delayed until the manometer indicates oxygen uptake by the phosphorus has ceased.

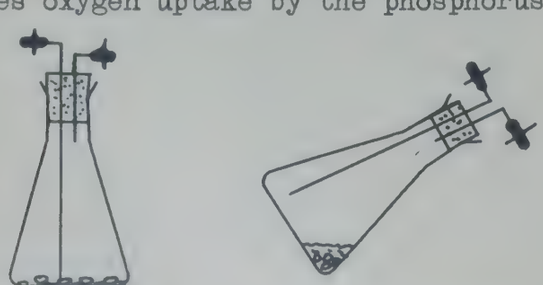

Fig. 10. Flasks for obtaining oxygen-free gases.

Heated copper filings are probably the most desirable for obtaining gas free from traces of O_2, especially if the gas contains CO_2 since no absorption of CO_2 occurs as with alkaline pyrogallol. However, such equipment is not always available and may occasionally be inadequate (for example, Keilin and Hartree (1943) found that nitrous oxides formed in the reaction inhibited the action of catalase).

A method which can serve under these circumstances is the following: To a stoppered Erlenmeyer flask (as indicated in Fig. 10) add 1 - 2 grams of yellow phosphorus and sufficient water to cover the phosphorus when tilted, but not sufficient to cover the phosphorus when the flask is level (see Fig. 10).

Fill the flask with the gas mixture (preferably by evacuation; see below), and expose the phosphorus by standing the flask in a level position. Leave in this manner for two to three days. The process may be shortened to 20 - 30 minutes by placing the flask in a water bath at 60°C (the phosphorus melts and must be shaken frequently). The phosphorus reacts with any oxygen present to produce phosphorus pentoxide which gradually dissolves in the water forming phosphoric acids. The oxygen free gas may then be placed in Warburg flasks by the evacuation method described below. Suction flasks are desirable when the gas mixtures are made by evacuation. With explosive gas mixtures (e.g., H_2) exposure of the phosphorus should be made slowly and the operator protected.

ALTERING GAS ATMOSPHERES

R. H. Burris

At times it is necessary to work in gas mixtures other than air. For this purpose one employs side arm flasks of the following form (Fig. 11) which, when the stopper of the side arm is appropriately turned, permit the exit of gas through the side arm. Gas mixtures are supplied through the opened stopcock of the closed arm of the manometer.

Fig. 11. Sidearm flask with gas-vent.

It is necessary to run about a liter of gas through the flask to assure that air has been displaced. By the use of a manifold arrangement an entire bank of flasks may be supplied with gas at one time. As an indication of the rate of gas flow a "U tube" is attached with rubber tubing to the sidearm stopper. The end of the glass "U" is pulled out into a capillary and dips below the surface of the water in the constant temperature bath; bubbles from the tube show the passage of gas.

When a uniform gas mixture is to be used routinely, e.g., 5% CO_2 95% O_2, it is convenient to purchase such a mixture in steel cylinders. When small quantities of a number of mixtures are necessary they may be prepared in stoppered bottles by filling the bottles with water and displacing measured quantities of water with the gases comprising the mixture--the water is displaced directly into a graduated cylinder for estimation of volume. Alternatively, the stoppered bottle may be evacuated, flushed with one of the component gases and then filled with the various gases to the desired pressures as indicated by a mercury manometer. The gas mixtures may be displaced from the bottles with water and passed through the Warburg flasks. (When the mixtures contain CO_2, the use of water as a displacing fluid will alter the composition of the mixture unless previously saturated with CO_2 at the pressure used).

If it is necessary to work with a large number of gas mixtures simultaneously, considerable labor is involved in making up the mixtures frequently. Also, when using expensive gases (e.g., purified helium) the cost may become prohibitive, or when a poisonous gas (e.g., CO) is used one does not wish to allow much of it in the laboratory atmosphere. To decrease the amount of gas necessary to flush a flask we have employed an evacuation procedure. Tubes in the form of a reversed h with a T at the top of the h are connected in series (a bank of 7 manometers may be treated at one time) as illustrated in Fig. 12 and attached with rubber tubing to both arms of the Warburg manometer. The Brodie fluid is lowered to within a few cm. of the bottom of the manometer columns, as it will rise during the evacuation. After making sure that the manometer stopcocks are open the screw clamp A is opened and the system is evacuated by means of a water aspirator until there is about 75 mm. Hg residual pressure, as indicated by mercury manometer C. Screw clamp A is now closed and clamp B opened; water in bottle D displaces gas from storage bottle E into the system until atmospheric pressure is reached. After two more evacuations and refillings the replacement of the original atmosphere is sufficiently complete, i.e., approximately 0.1% of the original gas remains. On the last filling the pressure is allowed to build up until it spills through the mercury manometer. The rubber tubing is then removed

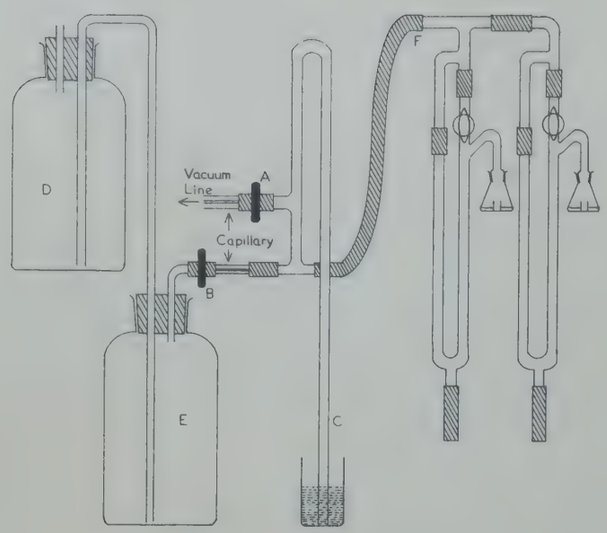

Fig. 12. Apparatus for altering the gas atmosphere by the evacuation method.

at F and the excess gas is lost from the flasks obviating the difficulties that would arise if a small vacuum were left to suck air into the flasks. The stopcocks on the manometers are closed immediately and the manometers placed on the bath; as the flasks warm up the stopcocks must be opened momentarily to release gas. If desired, the evacuation procedure may be performed in the constant temperature bath, though this generally presents no advantage.

When the system is under vacuum, the rubber tubing which serves as a reservoir for Brodie fluid will be compressed pushing the fluid up in the manometers. It is necessary to use tubing with reasonably heavy walls to minimize this effect. Although a vacuum better than 75 mm. H_g residual pressure can be readily obtained, its use is not recommende routinely because of the bubbling of the Brodie fluid that may occur. It is helpful to de-gas the Brodie fluid under vacuum before filling the manometers. Capillaries are introduced in the vacuum and gas lines to limit the rate of gas flow; sudden changes in pressure are not registered uniformly on the two columns of the Warburg manometer because the large volume of gas leaving or entering the Warburg flask must pass through the capillary tubing of the manometer. If the stopcock of the manometer is closed or plugged, vacuum is applied to one side of the manometer only, and fluid may be displaced into the evacuation line.

The method of adding gases by evacuation requires only a tenth to a twentieth as much gas as the flushing procedure, and one is always certain that every dead space in the most complicated flask has had its initial gas displaced by the desired gas mixture. Although the procedure was evolved originally to save valuable gases it has proved so convenient that we use it routinely.

METHODS OF PREPARING GAS MIXTURES

W. W. Umbreit

Displacement Procedures: If pure gases are available, mixtures may be prepared by the displacement of water or other fluid. One usually prepares flasks or bottles completely filled with the confining fluid, allows the various gases to enter until a given quantity of the fluid has been displaced. The examples below will clarify the actual procedure:

Example: Gas mixture required: 100 ml. of 20% O_2, 30% H_2, 50% N_2. Pure N_2 is added to displace 50 ml. of water from a flask completely filled with water. 20 ml. of water are displaced with O_2 and a further 30 ml. displaced with H_2. An alternative procedure, in case pure N_2 were not available would be: Add 50/.8 = 62.5 ml. air (of which 50 ml. is N_2, 12.5 ml. O_2), 7.5 ml. O_2 (20 ml. required in all less 12.5 ml. added with air equals 7.5 ml. still required), and 30 ml. H_2.

Displacement procedures are quite adequate, but one must be certain that the gas displacing the water is at atmospheric pressure, or at least that all gas added to form the mixture is supplied at the same pressure.

Evacuation Procedure: Atmospheric pressure is observed on a barometer. The gas bottle, in which the mixture is to be made, is attached to a manometer, as shown in Fig. 13. Connection A is closed (and kept closed throughout the operation), B is opened, and the flask is evacuated by attaching a water pump at C. Gases are added through C until the proper mixture is obtained. The procedure is best illustrated in the examples on the following page. Any shaped container of any volume may be employed with no alteration in the method of preparing the gas mixtures.

Example 1: Gas mixture required 20% O_2, 30% H_2, 50% N_2. Atmos. pressure = 748 mm. Hg. Using air for the nitrogen source (assuming 80% N_2 and 20% O_2 in air), one needs 50% of 748 mm = 374 mm. of N_2. To obtain this amount of N_2 from air, one needs 374/.8 = 467.5 mm. of air. Hence one would evacuate with the water aspirator until the mercury in the manometer stood at (748 - 467.5 =) 280.5 mm. 20% oxygen requires 20% of 748 mm. = 149.6 mm. However 93.5 mm. have already been added with the air, hence 56.1 mm. of pure oxygen are to be added. One then attaches an oxygen source to C, and admits oxygen until the level of mercury in the manometer reaches (280.5 - 56.1 =) 224.4. 30% hydrogen requires 30% x 748 mm. = 224.4 mm., hence one attaches a hydrogen source to C, and permits hydrogen to enter until the manometer reaches zero (224.4 - 224.4 mm.).

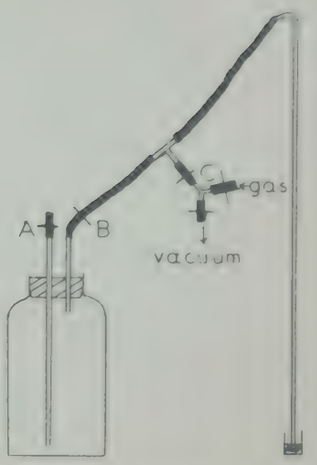

Fig. 13. Apparatus for preparing gas mixtures by evacuation and refill.

To prepare gas mixtures which contain no oxygen, evacuate the gas bottle, refill with one of the components of the gas mixture, repeat this process twice, and proceed from this point. This is illustrated in the two examples given below:

Example 1: Gas mixture required: 80% H_2, 5% CO_2, 15% N_2. Atmospheric pressure, 748 mm.; aspirator can evacuate to 720 mm. Evacuate to 720, return to zero with H_2; evacuate to 720, return to zero with H_2. Evacuate to 149.6 mm. (gas remaining in bottle is H_2); add CO_2 to 112.2 (from 149.6 - (0.05 x 748)), return to zero with pure N_2.

Example 2: Gas mixture required: 20% H_2, 20% N_2, 60% He. Atmospheric pressure, 748; aspirator can evacuate to 720 mm. Evacuate to 720, return to zero with H_2 or N_2 (Helium is expensive hence it is not employed in the flushing out process). Repeat twice. Evacuate to 598.4 (gas remaining in bottle is H_2 or N_2), return to 448.8 with N_2 (or H_2); return to zero with Helium.

THE USE OF CYANIDE AS AN INHIBITOR

W. W. Umbreit

Cyanide is a very useful inhibitor for the study of oxidations. In experiments with cyanide, however, special precautions must be observed if the inner cup of the manometric flask contains alkali for the absorption of CO_2. HCN is volatile and is rapidly absorbed by alkali. If the concentration of HCN is low all the cyanide distills into the center cup and no inhibition is observed. If the concentration of HCN is large, errors arise because the cyanide gradually decreases. Similar results have been reported for azide (Machlis, 1944).

These errors can (and must) be avoided if the center cup contains an alkali-cyanide mixture in which the concentration of free HCN is equal to the concentration in the experimental fluid.

The data of table XI enable one to determine the type of mixture one should employ in the center cup to absorb the CO_2 in the presence of cyanide (Warburg's "Direct Method", Chapter 1). In order to absorb CO_2 effectively the pH of the center cup must be 12 or above. Thus at pH 7 one cannot employ a concentration greater than M/1000 KCN (at pH 7, this means 0.99 x 10^{-3} M free HCN) without obtaining a gradual distillation of HCN into the center cup (since at pH 13 (center cup) even M/1 KCN only yields 0.14 x 10^{-3} M free HCN). However, for short time experiments, the actual loss of cyanide may be of little significance because its "volatility constant", i.e., the amount of HCN which escapes into the gas phase, is relatively low. The safest point, however, is to use cyanide concentrations which give equal amounts of free HCN in both the tissue and the alkali.

TABLE XI

Free Hydrocyanic Acid in Cyanide Solutions

pH	Conc. free HCN in "B" molar KCN	pH	Conc. free HCN in "B" molar KCN
5	B	9	0.58B
6	B	10	0.123B
6	0.999B	11	0.0138B
7	0.99B	12	0.0014B
8	0.875B	13	0.00014B

Derivation of Relationships Used in Table: KCN added to water is completely ionized at all concentrations below 10th molar (at M/10 it is 86% ionized). Hence the addition of KCN is equivalent to adding K^+ and CN^- ions. These react with the H^+ and OH^- ions, of the water to form, momentarily HCN and KOH but the HCN is almost unionized, hence can exist in the free state.

Adding KCN to water:

$$K^+ + CN^- + H^+ + OH^- \rightleftharpoons K^+ + OH^- + HCN$$

(a) $K_{\text{"hydrolysis"}} = (HCN)\ \cancel{(OH^-)(K^+)}/\cancel{(K^+)}(CN^-)(H^+)\cancel{(OH^-)}$

$$= \frac{(HCN)}{(CN^-)(H^+)}$$

This, it will be noted is the reciprocal of the acid dissociation constant, $K_a = (H^+)(CN^-)/HCN$

K_a is known to be (25°C): 7.2×10^{-10}

Hence $K_h = 1/7.2 \times 10^{-10} = 1.4 \times 10^9$

Hence at any pH, and with B molar cyanide the following relationships would hold.

(b) (CN) + HCN = B Mols ("B")

From equation (a)

$$K_h = HCN/(CN^-)(H^+) \quad \text{so} \quad (CN^-) = HCN/K_hH^+$$

From equation (b)

$$HCN/K_hH^+ + HCN = B$$

$$HCN + K_hH^+HCN = BK_hH^+$$

$$HCN(K_hH^+ + 1) = BK_hH^+$$

$$HCN = BK_hH^+/(K_hH^+ + 1)$$

(H^+) can be replaced by 10^{-pH}

Since, by definition of logarithms $x = 10^{\log x}$ thus $(H^+) = 10^{\log H^+}$ while from definition of pH; $-pH = \log H^+$ hence $(H^+) = 10^{-pH}$

$$HCN = BK_h \times 10^{-pH}/(K_h\ 10^{-pH} + 1) = B(1.4 \times 10^{(9 - pH)}/(1.4 \times 10^{(9 - pH)}_{+ 1})$$

Thus at pH 7

$$HCN = B(1.4 \times 10^{2})/(1.4 \times 10^{2} + 1) = B140/(140 + 1) = 0.99B$$

Thus, in a reaction flask containing 0.01 molar ($^{M}/_{100}$) KCN at pH 7, there is actually 0.99×10^{-2} mols. HCN. In the center cup one needs then, a concentration of 0.99×10^{-2}M cyanide. Krebs (1935) obtains this concentration by using 10 ml. 2N KCN with 0.2 ml. N KOH. The KOH concentration is thus: 0.02 N, which, assuming complete ionization, gives roughly a pH of 12. The cyanide concentration is 2 M and (from Table XI) the HCN concentration at this pH is ($1.4 \times 10^{-3} \times 2M =$) 2.8×10^{-3}, i.e., much less than is actually necessary for adequate counteraction of the HCN distillation. This is adequate for short time experiments, however, because the cyanide concentration in the flask is so high that a slight loss is negligible.

At lower levels of cyanide, i.e., 10^{-4}, Krebs (1935) uses 5 ml. 1 M KOH, 5 ml. 1 M KCN. If the external pH is 7, from Table XI, the tissue solution is 0.99×10^{-4} molar free HCN. The pH inside of the cup is (0.5 M KOH) close to 13, hence with 0.5 M KCN at pH 13, the concentration of free HCN is 0.7×10^{-4}. This is a relatively close relationship and one probably adequate for short time experiments.

A somewhat more convenient system for obtaining the requisite cyanide concentrations in the center cup is the following:

A 0.2 M solution (1.12% solution) of KOH is prepared. For practical purposes it may be considered that, when diluted with an equal volume of water, the pH will be close to 13. One then adds this solution, or dilutions from it to the center cup together with KCN as indicated in Table XII.

TABLE XII

Mixtures for Cyanide Inhibition Studies

In Center Cup		Approximate Concentration free HCN	Possible KCN conc. in tissue at pH 7*
Add 0.1 ml. KOH	Add 0.1 ml. KCN		
0.002M	2M	0.9×10^{-2}	10^{-2}
0.02M	2M	0.9×10^{-3}	10^{-3}
0.2M	2M	0.9×10^{-4}	10^{-4}
0.2M	0.2M	0.9×10^{-5}	10^{-5}
0.2M	0.02M	0.9×10^{-6}	10^{-6}

*For other pH values see Table XI.

ADDITION OF MATERIALS DURING THE COURSE OF THE REACTION

It is frequently desirable to add substances to reaction flasks during the course of the reaction. This is usually accomplished by using Warburg flasks equipped with one or more sidearms (Fig. 14). In tipping in materials from sidearms, wash the sidearm with some of the fluid in the main compartment of the flask and tip this also back into the main compartment. Only thus is quantitative transfer of the material in the sidearm obtained. When removing manometers and attached flasks from baths for such operations, place a finger over the open end of the manometer to prevent the rapid expansion or contraction due to temperature changes from either pushing out or sucking back the fluid in the manometers. When filling sidearms, do not fill to full capacity. If the sidearm is capable of holding 1 ml., it can best be employed for contents of 0.5 ml. or less. Not only is this to prevent any material from spilling over into the main compartment before the desired point, but also, if the sidearm is completely filled, the fluid in it has but little chance to come into equilibrium with the gas phase in the flask (since shaking does not alter its surface under these circumstances). When added to the main compartment it may thus take up or evolve gas very suddenly in reaching an equilibrium. Sudden

release or uptake of gas upon tipping in the sidearm, which does not continue at a similar rate, is usually due to a faulty equilibrium between the gas and the fluid in the sidearm.

It is also well to adjust the composition of the materials in the sidearm so that only one factor is altered upon the addition of its contents to the main compartment. For example, if one adds 0.5 ml. M/10 glucose to a bacterial suspension in M/50 phosphate buffer, one alters not only the glucose concentration, but the phosphate concentration as well.

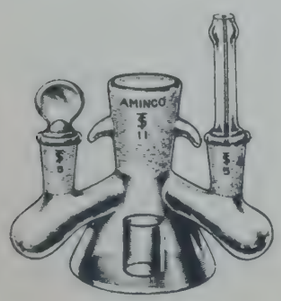

Fig. 14. Double sidearm Warburg flask.

Another method of adding materials to flasks with insufficient sidearms, employs "Keilin cups". These are small tubes (first described by Keilin, 1929) provided with a small hook (either of platinum wire or of glass) by which they may be hung from the edge of the center well. They can be dislodged by a careful jarring of the apparatus. It is possible (as pointed out by Dixon (1943)) to add more than one material by use of two "Keilin cups" with hooks of different length, one being released by a less vigorous jar of the apparatus than the other. It is probable, however, that the sidearm addition method is preferable whenever possible. The most useful, all purpose flask, as shown in Fig. 14, is one equipped with two sidearms, one of which is a gas vent arm.

ZERO POINTS

The zero point should be chosen so as to make maximum use of the graduated scale of the manometer. When gas uptakes are consistently measured, 250 mm. is a convenient point. When gas evolution is measured, 10 or 100 mm. may be used. The following method, using 150 mm. as the zero point is employed in the laboratory of Dr. Cohen:

In measuring gas uptake, the manometer fluid is set, with the stopcock open, near the bottom of the scale, as shown in Fig. 15. The stopcock is closed. Adjusting the fluid to the zero point (150 mm.) in the closed arm, raises the fluid in the open arm to near the top of the graduated scale. In measuring gas release, the opposite type of setting is employed as indicated in Fig. 15. This permits the use of the same zero point for both uptake and release with maximum use of the graduated scale.

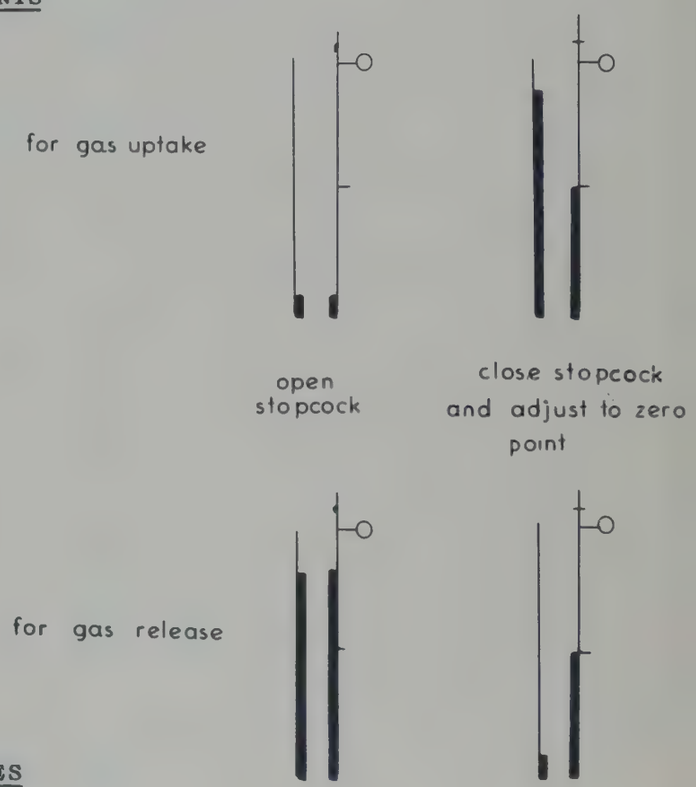

Fig. 15. Diagram illustrating the manometer setting to obtain maximum range.

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Chapter Six

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Chapter Six

DETERMINATION AND USE OF FLASK CONSTANTS

It is obviously of importance to know the exact volume of the flasks used in the Warburg instrument. Several methods of calibration are described below together with methods of calculating and using flask constants.

CALIBRATION WITH MERCURY

R. H. Burris

Although calibration with mercury may appear more laborious than other methods, it is the most accurate method available and is not particularly time consuming when a little skill is acquired with it.

With a diamond point scratch a permanent reference mark about 1 cm. above the flask-to-manometer ground joint. Weigh the Warburg flask empty. Fill the flask with clean mercury, and by using a capillary pipette with a bent tip tease any small air bubbles trapped at the sides and bottom of the flask to the surface. Place the flask on the dry manometer joint and seat the joint. If there is too much mercury or insufficient mercury to rise to the mark scratched on the capillary manometer, remove or add mercury with a capillary pipette until the mercury just reaches the mark when the joint is seated. Immediately plunge a thermometer into the flask of mercury and record the temperature. Weigh the flask and mercury.

Bore a hole in a rubber stopper to fit over the manometer ground joint, and put the stopper about half way onto the joint; this forms a well at the inlet to the manometer. Pour mercury quickly into this well with the manometer in an inverted position. This procedure minimizes the possibility of getting air bubbles in the mercury column. Tip the manometer to allow mercury to flow into the graduated arm of the manometer. Open the stopcock and allow the mercury to drop into contact with the stopcock. Remove the rubber stopper thus dumping the excess pool of mercury (it is convenient to work over a shallow box or tray lined with a sheet of paper to catch mercury). Tip the manometer until the mercury column coincides with the scratch mark and record the reading on the graduated manometer column. Weigh the mercury in a tared weighing bottle. Repeat the above procedure, this time introducing a shorter continuous column of mercury. Tip the entire column into the graduated manometer tube and record the mm. of tubing that it occupies. Weigh this mercury. Accept room temperature as a reasonably accurate measure of the temperature of the mercury.

Calculate the volume of the Warburg flask to the scratch mark by dividing the weight of mercury by its density at the recorded temperature. In the same manner find the volume of the manometer from the scratch mark to the recorded level, and the volume of the measured length of manometer tubing. Determine the volume of the manometer tubing per mm. length, and with this value correct the volume measured from the scratch mark to give the volume from the scratch mark to the 250 mm. mark. Calibration at the 250 mm. point is most convenient for studies of gas uptake. From the volume determined per mm. tubing length the calibration can be shifted to any other reference point that proves useful; the 50 mm. point is suitable when gas output is to be followed.

The sum of the flask volume and the manometer volume to the 250 mm. mark minus the liquid volume to be used in the flask is the V_g to be substituted in the equation to determine the flask constant. For density of mercury consult table XIII, page 51.

Schales (1944) has described an alternative procedure for calibration with mercury.

CALIBRATION WITH WATER

W. W. Umbreit

Perhaps the simplest and most convenient method of calibration is that of Vogler (1940) or Krebs (1929). This method determines the flask volume to within 5% and is especially convenient in the case of complicated attachments (such as Dixon-Keilin flasks, etc.).

TABLE XIII

Density of Mercury

Temperature °C	Density g/cc.
0	13.596
14	13.561
16	13.556
18	13.551
20	13.546
22	13.541
24	13.536
26	13.531
28	13.527
30	13.522

Procedure: Bring the manometer and flask to thermal equilibrium in the bath. Raise the manometer fluid in the closed arm of the manometer to 300 mm., close stopcock, and record reading on the open arm. Leaving the stopcock closed, bring the fluid in the closed arm to the reading "k" (usually the zero point, i.e., to 250 or 150 mm. If zero point were to be 250 mm., fluid would be brought down from 300 to 250 mm.). Record the reading on the open arm. The difference between the initial reading (closed arm at 300) and the final reading (closed arm at k) will enable one to calculate the pressure change in the process. This difference will be called "h_1".

Repeat the procedure with the flask filled with a known amount of water (W) and determine the drop "h_2" in the open arm of the manometer.

Let:

$$p_1 = h_1 - (300 - k) \qquad \text{and } p_2 = h_2 - (300 - k)$$

Then:

$$V_g = \frac{W\ p_2}{p_2 - p_1}$$

Theory: Let:

x (in mm^3.) = the volume of the capillary tube of the closed arm of the manometer over the distance from 300 to k.
P = atmospheric pressure.
V = V_g = volume of flask + manometer to the point k on the manometer.
W = volume of water added in the second procedure.

In the first procedure, the volume was increased by "x", hence

	Initial state (fluid at 300)		Final state (fluid at k)
(23)	$P\ (V - x)$	=	$(P - p_1)\ V$

Where p_1 = the observed change in pressure when the volume of the gas space is increased by "x". The value of p_1 is calculated from the observed drop (h_1) less the drop in the manometer fluid in the closed arm of the manometer (300 - k). This is done because the observed drop (h_1) is not due solely to the decreased pressure, but is also due to the lowering of the manometer fluid.

In the second procedure, the volume was increased by "x", hence

	Initial state (fluid at 300)		Final state (fluid at k)
(24)	$P\ (V - W - x)$	=	$(P - p_2)\ (V - W)$

Where p_2 = the observed change in pressure is calculated from the observations of the second procedure in the same manner as described for the first procedure.

From Equation (23): $Px = p_1V$ (23a)

From Equation (24): $Px = p_2V - p_2W$ (24a)

Substituting (23a) into (24a):

$$p_1V = p_2V - p_2W \quad \text{from which:}$$

$$V = V_g = \frac{W\ p_2}{p_2 - p_1}$$

Calibration details: To eliminate possible influences of changes in temperature and pressure on the partial pressure of the water, it is advisable to add a known amount of water in the first procedure. The accuracy of the method depends largely upon the value of h_2 which should be as large as possible. The amount of water to be added can be readily determined after the data of the first procedure are available, and is roughly two-thirds of the flask volume. One of the advantages of this method is that the ease with which it is done permits several determinations of the flask constant and thus permits calculation of the error attached to the constant.

Example: A double sidearm flask was supplied with 1 ml. water, shaken in bath at 28°C., 20 minutes. First procedure: Reading of open arm when closed arm was at 300 mm. = 29.2; Reading at 250 mm. = 17.3. Thus $h_1 = 11.9$, $p_1 = 11.9 - 5 = 6.9$. Five ml. more water added (6 ml. in all). Shaken at 28°C for 20 minutes. Second procedure: Reading at 300 = 28.75; Reading at 250 = 13.9. Thus $h_2 = 14.85$, $p_2 = 14.85 - 5 = 9.85$.

$$\text{Volume with 1 ml. water} = \frac{5 \times 9.85}{9.85 - 6.9} = 16.69 \text{ ml.}$$

Volume of flask empty = 16.69 + 1.0 = 17.69 ml.

Repeat calibration: 17.4 ml., 17.65 ml. 17.73 ml. Calibration with mercury: 17.80 ml.

CALIBRATION BY THE ADDITION OR REMOVAL OF A KNOWN QUANTITY OF GAS

The removal of known quantities of gases as a means of calibration is described by Dixon (1943) and in Chapter 7. One may also generate known quantities of gas (e.g., CO_2 from bicarbonate and acid) and determine the change in manometer level. Since "x" is now known, the V_g of the flask can be calculated.

FLASK CONSTANTS

As derived in Chapter 1, the relation between the difference in reading observed on the manometer (h) and the gas change within the system (x) is:

$$x = h\left[\frac{V_g \frac{273}{T} + V_f\alpha}{P_o}\right] = hk \quad \text{where } k = \left[\frac{V_g \frac{273}{T} + V_f\alpha}{P_o}\right]$$

Since for any given experimental condition, the gas volume of the flask (V_g) the fluid volume of the flask (V_f), the temperature (T), the solubility of the gas in the fluid (α), and the pressure (in mm. of manometer fluid) of 1 atmosphere (P_o) are all constant, the value "k" (the "flask constant") is constant for any given set of experimental conditions and varies only as the experimental conditions vary.

FACTORS FOR DIFFERENT VOLUMES OF FLUID IN THE FLASKS

One can, of course, calculate the constant to be employed for any set of experimental conditions from the equation above. But it is frequently convenient to merely alter the constant from one experimental condition to another without recalculating by means of the entire equation above. For increasing volumes of fluid in the flask, the relationship between the presence of 1 ml. more fluid in the flask, and the flask constant is very simple: (we will define this value as Δ_{1000} since 1 ml. = 1000 µl.)

The presence of 1 ml. more fluid in a flask will result in a factor k' such that:

$$k' - k = \Delta_{1000} = \frac{(V_g - 1000)\frac{273}{T} + \alpha(V_f + 1000)}{P_o} - \frac{V_g \frac{273}{T} + \alpha V_f}{P_o} = \frac{-1000\frac{273}{T} + 1000\alpha}{P_o}$$

(represents k', i.e., flask with 1 ml. more fluid in it)

(represents k, i.e., original flask)

Thus, Δ_{1000} is independent of V_g or V_f and dependent only on α and T. For any temperature, Δ_{1000} is easily calculated. Thus for k_{O_2}:

Temperature °C	Δ_{1000}*
20	- 0.091
28	- 0.088
33	- 0.087
38	- 0.085
43	- 0.084
48	- 0.083

*For each ml. of additional fluid in the flask the factor changes by this amount.

Thus any amount of non-gaseous matter can be added to the flask in the run of an experiment, if its volume is known, without lengthy calculations.

On this principle also estimations of the inaccuracy due to the addition of solids of unknown volume (filter paper, glass rods, tissue slices, etc.) can be made easily.

CALCULATIONS OF FACTORS FROM $K_{O\ ml.}$

It will be noted that since:

$$k = \frac{V_g \frac{273}{T} + \alpha V_f}{P_o}$$

the k for 0 ml. fluid is the same independent of the gas involved, since at 0 ml. fluid, the factor becomes

$$k_{0\text{-ml.}} = \frac{V_g \frac{273}{T}}{P_o}$$

Furthermore, the previous section has shown that for each ml. of fluid added the factor changes by a constant amount. Hence the following table may be employed:

Temp. C°	$k_{0\text{-ml.}}$	$\Delta_{ml.\ oxygen}$
37	V_g x .0880	0.085
30	.0900	0.088
25	.0910	0.091
20	.0931	0.091

For example, the k_{O_2} for a flask having a total volume of 13.5 ml. and containing 1.2 ml. fluid would be: (at 28°C)

$$k_{O\text{-ml.}} = 13.5 \times 0.0910 = 1.23 \qquad 1.2 \text{ ml. fluid} \times 0.088 = 0.106$$

$$k_{O_2} = 1.23 - 0.106 = 1.124$$

For a fluid volume of 2 ml. the factor would be

$$k_{O_2} = 1.23 - 0.176 = 1.054$$

And similarly for other fluid volumes.

MacLeod and Summerson (1940) have described a graphical method of determining flask constants. If the constant at a given temperature calculated by the usual equation for a liquid volume of 0 ml. (V_f term drops out) and any other volume (e.g., 3 ml.) is calculated and the constants are connected by a straight line on a graph with ml. liquid volume as abscissa and flask constant as ordinate, the constant for any liquid volume can be taken from the line. With a series of lines (the lines for a given temperature have the same slope for any flask, but a different intercept for each flask), for 10° temperature intervals, flask constants at any liquid volume and temperature can be obtained by inspection.

DESIGN OF WARBURG FLASKS

Two shapes of flasks are in common use. These are illustrated in Fig. 16. Flasks of type A (with angle edges) tip less easily when detached from the manometer and are suitable for use on manometric instruments which (as illustrated in Fig. 2) employ a reciprocating motion whereby the flask is moved to and fro in the same plane. When used on the type of instrument in which the pivot of the manometer is at the bottom and the flask is rocked back and forth, tissue tends to collect at the edge of the flask. Type B (with rounded edges) may be used equally well with both types of shaking devices, but, because the bottom is not flat, it tends to tip more easily. Type A is also preferred for irradiation work.

Fig. 16. Different shapes of Warburg flasks.

An additional modification has been suggested by Dr. Cohen. If the center well is slightly constricted at the top (as shown in "B", Fig. 16), splashing or "creeping" of alkali into the main compartment is largely prevented.

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Chapter Seven

THE DIFFERENTIAL MANOMETER WITH SPECIAL REFERENCE TO ITS USE IN STUDIES OF PHOTOSYNTHESIS

J. F. Stauffer

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Chapter Seven

THE DIFFERENTIAL MANOMETER WITH SPECIAL REFERENCE TO ITS USE IN STUDIES OF PHOTOSYNTHESIS

THEORY

The differential respirometer was introduced by Barcroft (1908), and it is often called the "Barcroft respirometer", or "Barcroft manometer". The term respirometer is somewhat misleading however, since this type of apparatus has been used in a number of laboratories for measuring the rate of photosynthesis. It will suffice to point out that any type of apparatus designed to measure the uptake of a gas in terms of the change in the reading of a manometer can serve equally well to measure the evolution of the gas. Of course, in either case all conditions affecting the gas exchange must be taken into consideration.

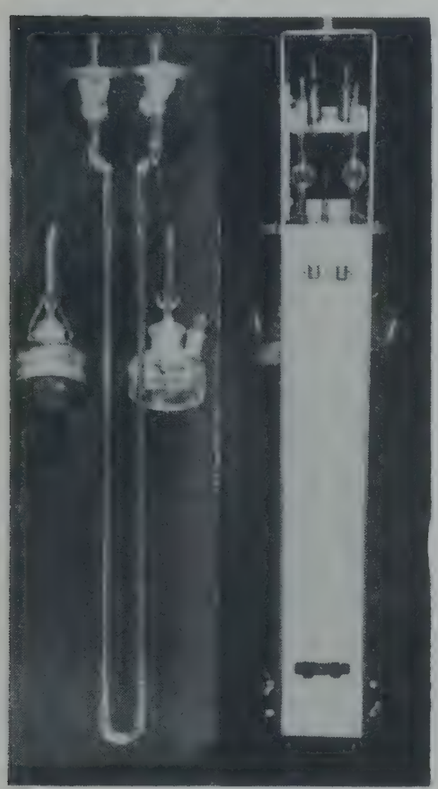


Fig. 17. The Differential Manometer.

The differential respirometer is shown in Fig. 17. It is essentially a closed system formed of two flasks connected by a manometer. In practice the volumes of liquid and gas on both sides of the manometer are the same. One flask contains the cells or tissue and is called the reaction vessel (on the right, R). The other flask (on the left, L), free from cells or tissue, is called the compensation vessel, i.e., it serves to compensate for changes in temperature and barometric pressure during the course of an experiment. It is readily understood that a change in temperature will have the same effect (on the volume and pressure, and on the solubility of the gas being measured) in each flask and thus will produce no change in the height of the manometer liquid. Also, the use of the compensation vessel to form a closed system makes the manometer readings independent of any changes in barometric pressure which may occur during an experiment, i.e., a thermobarometer is unnecessary.

CONSTRUCTION AND MOUNTING

The respirometer is constructed entirely of pyrex glass, preferably with standard interchangeable ground glass joints for the stop-cocks and for the connection between the flasks and the limbs of the manometer. The manometer, including the stop-cock connections and the limbs carrying the flasks, is of thick-walled capillary tubing of uniform bore. The upright portion of the manometer is about 45 cm. in length. Each side is graduated at the same level in millimeters, with numbered centimeter graduations, over a 30 cm. portion. The two symmetrical halves of the respirometer are constructed as nearly alike as possible in respect to the internal diameter of the capillary tubes and gas volume. There is no restriction on the design of the flasks provided the gas exchange of the cells or tissue is not hindered. Many laboratories use the standard Warburg flasks with side arms and center well. For photosynthesis measurements, flasks similar to that represented in Fig. 18 are used. Generally, the flasks do not have a volume of more than 40 ml.

The respirometer is mounted on an inverted L-shaped wood or metal support which in turn is attached to a suitable shaking device. As mounted, the graduated portions of the manometer tubes are vertical. The flasks are side-by-side and submerged at least 2 cm. beyond their necks in a constant temperature bath.

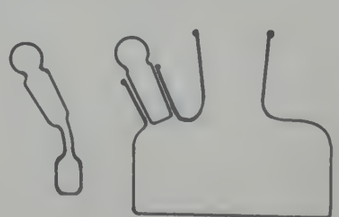


Fig. 18. Flask for use in photosynthesis measurements. The paddle attached to the vent plug facilitates stirring (Emerson and Lewis, 1941).

Three types of shaking devices are in use at present: (1) the older method of rocking the vessels from side to side; (2) the more recent method of short side-to-side swings (Dixon, 1943); (3) a special type of shaking device whereby the flasks are rotated a few millimeters off-center (Warburg and Negelein, 1922). The second type of shaking device is common in all respiration laboratories; the third type is in use in photosynthesis laboratories, especially where the tissue or cell suspension must be irradiated, and the quantity of radiant energy absorbed determined. Regardless of the type, all of these shaking devices are constructed to allow considerable variation in rate of shaking and in the amplitude of the swing, or rotation, of the flasks.

THEORY

The theory of the apparatus has been described and elaborated on a number of times since the original account by Barcroft (1908). The most complete description of the theory for use in measurements of photosynthesis and respiration is given by Warburg and Negelein (1922). A complete account of the theory suitable for use in respiration studies is given by Warburg (1923). Dixon (1943) has also published a very complete description, together with several extensions and modifications. The following account is based on those of Dixon and Warburg and Negelein.

Let: R = Reaction vessel.
L = Compensation vessel.
V_g = µl. of gas in the reaction-vessel side of the manometer.
V'_g = µl. of gas in the compensation-vessel side of the manometer.
V_f = µl. of liquid in the reaction vessel (including the tissue).
V'_f = µl. of liquid in the compensation vessel.
A = Cross-sectional area of the manometer capillary, in mm^2.
h = Manometer reading in mm. (the difference in height of the manometer liquid in the limbs of the manometer).
P_o = Normal pressure (760 mm. Hg), in mm. manometer liquid.
P = Atmospheric pressure (corrected), in mm. of manometer liquid.
ΔP = Increase in pressure of the confined gas in the reaction vessel.
$\Delta P'$ = Increase in pressure of the confined gas in the compensation vessel.
p = Pressure of water vapor in the gas spaces, in mm. manometer liquid.
T_o = 273° Absolute.
T = Temperature of the gas and liquid in the vessels in absolute degrees.
$\underline{\alpha}$ = Solubility coefficient of the gas produced or absorbed (µl. per µl. of liquid).

Suppose the apparatus contains the same volumes of gas and water on both sides of the manometer, and that the manometer liquid is at the same height in the two limbs of the manometer. The amount of gas (at 0°C, 273° Abs., and 760 mm. Hg; or NTP) in each gas space is:

$$\text{For } R = V_g \frac{273}{T} \frac{P - p}{P_o} \qquad \text{For } L = V'_g \frac{273}{T} \frac{P - p}{P_o} \quad (25, 25')$$

If x amount of a gas is evolved in R, the manometer reading will be h. The increase in volume of the gas space in R is then $\frac{h}{2}A$; the decrease in volume of the gas space in L is also $\frac{h}{2}A$. But h does not indicate the true value of ΔP of the gas in R because of the compensatory effect of the rise of pressure in L. In other words, both pressure and volume increase in R while the pressure increases and the volume decreases in L. Taking these changes into consideration, the final volumes of the gas spaces will be:

$$\text{for } R = \left(V_g + \frac{h}{2}A\right)\left(\frac{P + \Delta P - p}{P_o}\right) \qquad \text{for } L = \left(V'_g - \frac{h}{2}A\right)\left(\frac{P + \Delta P' - p}{P_o}\right) \quad (26, 26')$$

Since the pressure has increased in each vessel, the increase in the amount of gas dissolved in the liquid must be taken into account. It is:

$$\text{for } R = V_f \underline{\alpha} \frac{\Delta P}{P_o} \qquad \text{for } L = V'_f \underline{\alpha} \frac{\Delta P'}{P_o} \quad (27, 27')$$

Summarizing: The amount of gas, x, produced in R is equal to the sum of the final volume of the gas space and the increase in the amount of the gas dissolved in the water less the original volume of the gas space; or,

$$x = (V_g + \frac{h}{2}A)(\frac{273}{T}\frac{P + \Delta P - p}{P_o}) + (V_f \alpha \frac{\Delta P}{P_o}) - (V_g \frac{273}{T}\frac{P - p}{P_o})$$

$$\text{and } x = V_g\frac{273}{T}\frac{\Delta P}{P_o} + \frac{h}{2}A\frac{273}{T}\frac{P + \Delta P - p}{P_o} + V_f \alpha\frac{\Delta P}{P_o}$$

$$\text{and } x = \Delta P\left[\frac{V_g\frac{273}{T} + V_f \alpha}{P_o} + \frac{A}{2}\frac{273}{T}\frac{(P - p)\frac{h}{\Delta P} + h}{P_o}\right] \qquad (28)$$

On the other hand, no gas was produced in L; but the changes in volume and pressure must be accounted for in arriving at a value for P in order to solve equation (28).

Thus,

$$0 = (V'_g - \frac{h}{2}A)\left[\frac{273}{T}\frac{P + \Delta P' - p}{P_o}\right] + \left[V'_f \alpha\frac{\Delta P'}{P_o}\right] - \left[V'_g\frac{273}{T}\frac{P - p}{P_o}\right]$$

$$\text{or, } 0 = V'_g\frac{273}{T}\frac{\Delta P'}{P_o} - \frac{h}{2}A\frac{273}{T}\frac{P + \Delta P' - p}{P_o} + V'_f \alpha\frac{\Delta P'}{P_o}$$

$$\text{or, } 0 = \Delta P'\left[\frac{V'_g\frac{273}{T} + V'_f \alpha}{P_o} - \frac{A}{2}\frac{273}{T}\frac{(P - p)\frac{h}{\Delta P'} + h}{P_o}\right]$$

$$\text{and } \Delta P' = h\left[\frac{\frac{A}{2}\frac{273}{T}(P + \Delta P' - p)}{V'_g\frac{273}{T} + V'_f \alpha}\right] \qquad (29)$$

As the pressure was originally the same on both sides of the manometer and as the pressure has increased by ΔP and $\Delta P'$ in R and L respectively, the difference in pressure as measured on the manometer is,

$$h = \Delta P - \Delta P'$$

$$\text{Or}^*, \Delta P = h + \Delta P' \qquad (30)$$

Substituting $\Delta P'$ of equation (29) in equation (30),

$$P = h + h\left[\frac{\frac{A}{2}\frac{273}{T}(P + \Delta P' - p)}{V'_g\frac{273}{T} + V'_f \alpha}\right]$$

$$\text{or, } P = h\left[1 + \frac{\frac{A}{2}\frac{273}{T}(P + \Delta P' - p)}{V'_g\frac{273}{T} + V'_f \alpha}\right]$$

And substituting this value of ΔP in equation (28) results in the complete expression for the differential respirometer:

$$x = h\left[1 + \frac{\frac{A}{2}\frac{273}{T}(P + \Delta P' - p)}{V'_g\frac{273}{T} + V'_f \alpha}\right] x \left[\frac{V_g\frac{273}{T} + V_f \alpha}{P_o} + \frac{A}{2}\frac{273}{T}\frac{(P - p)\frac{h}{\Delta P} + h}{P_o}\right] \qquad (31)$$

*This equation is for respirometers with vertical manometers. If the manometer is tilted, then $\Delta P = h \cos \theta + \Delta P'$ where θ is the angle of tilt.

For the usual type of differential respirometer V_g (and V'_g) is about 30,000 µl., V_f (and V'_f) is about 3,000 µl., and A is always less than 0.5 mm^2; ΔP (and $\Delta P'$) will probably never differ from h by more than 50%; the maximum value of h is 300 mm; P will rarely be less than 95% of P_o; T will rarely be greater than 37.5°C.; p will be less than 5% of P; also, $V_f \alpha$ (and $V'_f \alpha$) is not more than 5% of the value of V_g (and V'_g), even for the very soluble CO_2 gas.

If it is assumed that these various quantities have their maximum values as stated above, by substituting in equation (31) it is found that,

$$x = h(1 + 0.07)\ (2.9 + 0.13)$$

This shows that the second term is about 1/14 of the first and the fourth term is about 1/20 of the third. Such a trial calculation proves that it is permissible to substitute P for $(P + \Delta P' - p)$, to omit $V'_f \alpha$ in the second term, and to omit the last half of the fourth term of equation (31) without introducing an error of more than 1%. Thus, we obtain the simplified equation,

$$x = h\left[\left(1 + \frac{A\ P_o}{2\ V'_g}\right)\left(\frac{V_g \frac{273}{T} + V_f \alpha}{P_o} + \frac{A}{2}\frac{273}{T}\right)\right] \qquad (32)$$

where the product of the terms within the brackets is the "constant" of the respirometer. To obtain the volume, x, of the gas evolved at NTP it is necessary only to multiply the value of the constant by the manometer reading, h.

Dixon (1943) has pointed out that a description of the theory, such as that given above, which assumes that the whole apparatus is at the temperature T actually reduces the error of the constant as determined by equation (32); furthermore, the use of p at T instead of at room temperature (as should be the case) introduces an error of less than 0.5% in the constant. It was also assumed that the gas spaces were filled with the same kind of gas as that evolved. It might be supposed that the pressure of a second gas would affect the constant due to the fact that with the increase in volume, $\frac{h}{2}A$, the partial pressure of the second gas would be reduced and some of the gas would pass from the liquid into the gas space. However, the reduction in partial pressure of a contained gas due to the increase in volume is very small. Even for a relatively high concentration of a very soluble gas, i.e., 10% CO_2, the error introduced is less than 0.1%; the presence of 80% O_2 has even less effect.

CALIBRATION

The constant of the differential manometer may be arrived at by three methods: (1) by calculation, using the simplified equation; (2) by the Münzer and Neumann method and (3) by liberating or absorbing a known amount of gas in the reaction vessel by means of a chemical reaction. The first two methods are preferred by most workers, although the third has certain advantages. It cannot be too strongly emphasized that, whichever method is employed, the conditions under which the constant is determined must be the same as those which will prevail during the course of experimentation. If not, a correction must be applied.

Determining the constant by calculation: The usual case is to calculate the constant for O_2, i.e., $K_{O_2}(NTP)$. 'A' is determined by running in sufficient mercury to form a 100-150 mm. column in the graduated portion of one side of the manometer. While holding the manometer by its ends to avoid a change in temperature, measure the length of the mercury column in 3-4 positions. Run the mercury into the other side of the manometer and repeat the measurements. The length of the column in the several positions serves as a measure of the uniformity of the bore of the capillary tubing. The volume of the mercury column is calculated from the weight and density of the mercury at the temperature of the room. 'A' then equals the volume in µl. divided by the average length in mm. The A values for both sides of the manometer should agree to within 5%; if not, it is desirable to check the K_{O_2} obtained by this method with one of the other two methods; method (2) is readily adaptable to determining the K_{O_2} value for different values of h, which covers this variation in cross-sectional area of the manometer tubes.

To determine the volumes of the vessels and their manometer tubes: An index mark is made on each manometer limb about 1 cm. above the ground-glass vessel-joint. Attach a small funnel to the stop-cock capillary with a 25-40 cm. length of rubber tubing. While manipulating the manometer in an inverted position, add mercury through the stop-cock capillary until one meniscus coincides with the index mark and the other meniscus coincides with the "zero" on the graduated portion of the manometer (the 15 cm. graduation). Of course, there must not be any trapped bubbles of air. The stop-cock is now closed and the mercury in the manometer limb shaken out and weighed. The volume of the mercury is determined from its weight and density, and it represents the volume of the manometer limb from the "zero" of the manometer to the "index mark". The same procedure is followed to determine the volume of the other manometer limb. The volumes of the flasks, which have been labeled R and L respectively, are determined at the temperature of the bath. Using a bit of filter paper held with curved forceps, or a pipe cleaner, to remove bubbles of trapped air, fill each flask with sufficient mercury so that the mercury is forced up to the "index mark" on the manometer limb when the flask is attached to its manometer limb. Ordinarily this requires time and patience. The mercury-filled vessel is placed in a shallow dish (a tea-glass coaster works very well) and the whole supported in the bath with the neck of the vessel projecting above the water. Allow time for temperature equilibration. Take hold of the coaster and neck of the flask, and carefully work the neck of the flask onto the ungreased joint of its capillary limb. When this operation is properly carried out no mercury is trapped in the ground-glass joint. Usually the meniscus of the mercury does not coincide with the "index mark" on the first trial. Return the coaster-supported flask to the bath, and add or remove mercury from the flask with a capillary-tipped eye-dropper. After allowing time for temperature equilibration, again attach the flask to its manometer limb for another check on the coincidence of the mercury meniscus and "index mark". This is obtained by repeating the above procedure. The mercury is then weighed and its volume determined at the temperature of the bath. The main sources of error in this determination are: Air is often trapped below the ground-glass joint. It can be removed by rocking the top of the respirometer from side to side. The temperature of the glass and mercury may change considerably while the vessel is being attached to the manometer unless this operation is carried out quickly. Avoid contact between flask and hands as much as possible. Finally, check the final coincidence of the mercury meniscus and "index mark" by submerging the end of the manometer limb with the attached flask filled with mercury in the bath.

By adding the volume of the vessel to the corresponding manometer limb volume, the total gas volume of one side of the respirometer is obtained. Determine the total gas volume of the other side in the same manner. These two gas volumes should be equal to within 0.1%. In most instances they will be unequal in volume. While it may help to switch the flasks, the quicker procedure is to pair flask and limb of manometer so that the volume of the reaction-vessel side of the respirometer is the smaller. Glass beads can then be added to the compensation-vessel side to obtain the same gas volume. The requisite volume of glass beads can be measured out by adding beads to water in a partially filled burette until the necessary volume is obtained. This "pairing" is unnecessary if it makes no difference which limb of the manometer carries the reaction vessel.

The gas volume of each side can now be obtained by subtracting the volume of the liquid (including that in the side arms and center well, and the volume of the tissue) which will be used in the flasks from the total gas volume.

The density of the manometric liquid, e.g., iso-caproic acid, is determined at the temperature of the room by means of a pycnometer. P_o can then be defined in terms of mm. of manometric liquid.

The absorption coefficient, α, of oxygen in the liquid must be obtained from a table of "α values" (see Chapter I), or determined by actual experiment. For most purposes the α for oxygen in water is sufficiently accurate. 'α' had best be determined or obtained from the literature for liquids other than water if the volume of liquid in the flask is more than 10 ml. It is possible to calculate the α of oxygen, carbon dioxide, and nitrogen from the data of Geffken (1904) for salt solutions.

Having obtained the above data, the K_{O_2} of the respirometer may be calculated with the use of equation (32). The example below may be of help.

Details of Calibration: (a) General considerations: Room and bath temperatures, 25°C; iso-caproic acid as manometer liquid; flasks of Warburg type with two sidearms; volume of liquid in each flask, 4.2 ml. (3.0 ml. of nutrient solution, 0.5 ml. 5% glucose in each sidearm, 0.2 ml. 20% KOH in center well). The reaction vessel is to contain 100 µl. (0.1 ml.) of algal cells suspended in 3,000 µl. of nutrient solution.

(b) Determination of A.

Average length of Hg column in left side of manometer = 105.8 mm.
Average length of Hg column in right side of manometer = 107.6 mm.
Weight of mercury = 606.4 mg.

$$A = \frac{606.4}{106.7 \times 13.53} = 0.42 \text{ mm.}^2$$

(c) Determination of V_g and V'_g:

Wt. of Hg filling right flask and manometer limb = 251.3 gms.
Wt. of Hg filling left flask and manometer limb = 261.6 gms.

$$\text{Then } V_g = \frac{251.3 \times 1000}{13.53} - 4{,}200 = 14{,}372 \text{ µl.}$$

$$\text{And } V'_g = \frac{261.6 \times 1000}{13.53} - 4{,}200 = 15{,}134 \text{ µl.}$$

To reduce V'_g to the same volume as V_g, 760 µl. of glass beads are added to the left (compensation) vessel.

(d) Determination of P_o:

Wt. of 5.00 ml. of iso-caproic acid in a previously calibrated pycnometer = 4.605 gms.
Density of the acid = 0.921

$$\text{Then } P_o = \frac{760 \times 13.53}{0.921} = 11{,}164 \text{ mm.}$$

(e) The absorption coefficient, α, of O_2 = 0.030

(f) Calculation of K_{O_2}:

$$K_{O_2} = h\left[\left(1 + \frac{0.42 \times 11{,}164}{2 \times 14{,}373}\right)\left(\frac{\frac{14{,}373 \times 273}{298} + 4{,}200 \times 0.030}{11{,}164} + \frac{0.42 \times 273}{2 \times 298}\right)\right]$$

and $X_{O_2} = h(1.61)$.

Determining the constant by the Münzer and Neumann Method: This method (Münzer and Neumann, 1917) requires a carefully calibrated 1 ml. pipette provided with a leveling bulb containing mercury, a calibration manometer of 3 mm. glass tubing filled with kerosene or light paraffin oil colored with a dye (Sudan IV is satisfactory), and a capillary glass manifold connecting the reaction-vessel side of the respirometer with the pipette and calibration manometer and bearing a stop-cock at its free end. The set-up is illustrated diagrammatically in Fig. 19.

As arranged for a calibration, the set-up is much more compact than indicated by the diagram on p. 62. The pipette with its leveling bulb, the manometer, and the manifold can be attached by means of spring clips to an inverted L-shaped metal rod which in turn is attached to the respirometer support. Such an arrangement permits the apparatus to be placed on the shaker with the respirometer vessels, the pipette, and the lower portion of the calibration manometer immersed in a glass-fronted, constant temperature water bath. A suitable water bath may be improvised from a large bell-jar.

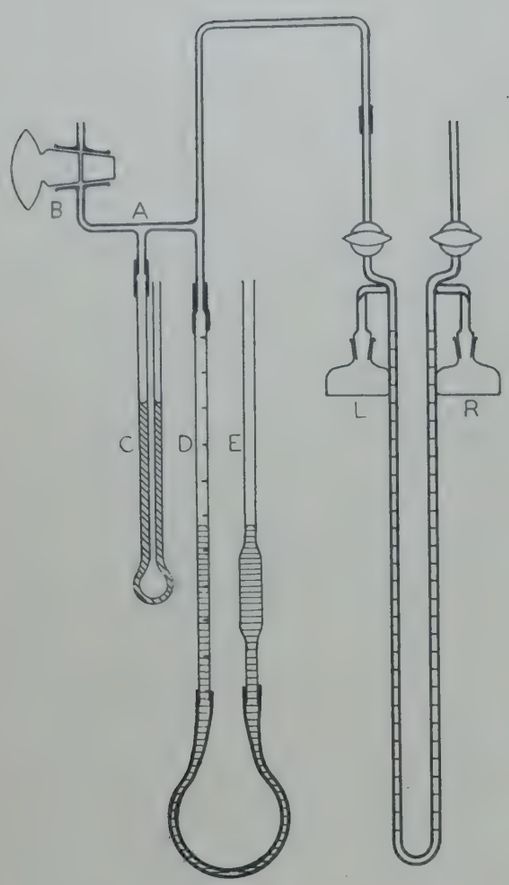


Fig. 19. Apparatus for Calibration by the Münzer-Neumann Method. A, manifold; B, manifold stop-cock; C, calibration manometer; D, 1 ml. pipette; E, leveling bulb; L, reaction vessel; R, compensation vessel.

After having thoroughly cleaned and dried the vessels and manometer capillary of the respirometer (see Chapter 5), pipette into the respirometer capillary sufficient manometer liquid to fill the manometer limbs to, or approximately to, their 15 cm. marks when the manometer is in a vertical position. Attach the holder bearing the manifold, etc., to the respirometer support. Add sufficient mercury to the leveling bulb to allow for the displacement of the entire gas volume of the pipette. Add sufficient colored paraffin oil to the manometer to bring the level of the oil about 10 cm. below the opening into the manifold. Pipette into the respirometer vessels the correct volume of the liquid or liquids (include that in the sidearms, but substitute water for the KOH in the center wall) which is to be used in determining the gas exchange of cells or tissue. Clean and replace the grease in the stop-cocks of the manometer and manifold. Attach the apparatus to the shaker with the vessels, etc., submerged in the constant temperature water bath. Do not attach the manifold to the respirometer, or close any of the stop-cocks. Allow 10-15 minutes for temperature equilibration. The manifold is now connected to the reaction-vessel side of the respirometer. After closing the stop-cock of the manifold, alternately raise and lower the mercury in the pipette to mix the air in the apparatus. Repeat this operation several times over a period of 10-15 minutes. It is not necessary to shake the apparatus while this equilibration is being carried out.

The manifold stop-cock is now opened, and the manometer and calibration manometer are checked for 'zero' reading. The following data are recorded: P (in mm. Hg at T°C.), room temperature, bath temperature, pipette reading and the height of the liquid in the right and left sides of the respirometer capillary. The manifold stop-cock and the stop-cock of the compensation-vessel side of the respirometer are now closed. From 60-100 µl. of gas is withdrawn from the respirometer by lowering the mercury in the pipette. The stop-cock of the reaction-vessel side of the respirometer is now closed, and the calibration manometer is again brought to 'zero' by raising the mercury in the pipette. At this point, it is best to allow the apparatus to stand for a few minutes to allow for restoration of equilibrium between the gases in the gas spaces and liquids in the vessels of the respirometer. After checking the 'zero' of the calibration manometer, record the height of the liquid in the right and left sides of the respirometer manometer and the pipette reading. The stop-cock of the reaction-vessel side of the respirometer is now opened, and the mercury in the pipette adjusted to its original level. The respirometer and calibration manometer should be restored to their 'zero' levels. If they are not, a leak, a change in temperature, or a change in barometric pressure during the calibration is indicated.

The above procedure is carried out several times, and the ratio of the volume of gas removed (or added) to h is calculated. Usually these agree to within 1%. The average ratio obtained is then substituted in the following equation in order to correct the value of the constant obtained under the conditions of calibration to 0°C. and 760 mm.

$$K_c = \left(\frac{\text{Vol. of gas}}{h}\right)\left(\frac{273}{T_c}\right)\left(\frac{P_c - p_c}{P_o}\right) \qquad (33)$$

where the subscript c refers to the value obtaining under conditions of calibration.

This equation expresses the relation between the µl. of gas (NTP) removed, or added, and the reading of the respirometer.

It might be questioned if this type of calibration, with the vessels containing water and air, holds for experimental liquids and gas mixtures. By choosing an α value for oxygen in water, it can be shown that the value of $V_f \alpha$ in those cases where V_f is of the order of 3000-5000 µl. is practically negligible in calculating k_{O_2} by use of the simplified equation. The $\alpha\ O_2$ for experimental liquids will not be greater, hence their presence in the vessels will not alter the value of K_c. Then the results of the calibration can be applied directly to the determination of oxygen. The above statements, however, do not apply to the very soluble CO_2 gas. In this case the calibration had best be made using the simplified equation.

As described above in the presentation of the theory, K_c does not vary with the temperature. On the other hand, if the $V_f \alpha$ term in the simplified equation is omitted the constant becomes exactly proportional to $1/T$. Then, to use the respirometer at an experimental temperature, $T_{exp.}$, which differs from T_o, a correction must be applied. Thus,

$$K_{exp.} = K_c \frac{T_c}{T_{exp.}}$$

Under experimental conditions, changes in barometric pressure and in the vapor pressure of water usually occur. Then, since K_c is inversely proportional to gas pressure,

$$K_{exp.} = K_c \left[\frac{T_c}{T_{exp.}} \frac{P_c - p_c}{P_{exp.} - p_{exp.}} \right]$$

and correcting $K_{exp.}$ to give µl. of dry gas at NTP,

$$K_{exp.} = K_c \left[\left(\frac{T_c}{T_{exp.}} \right) \left(\frac{273}{T_c} \right) \left(\frac{P_c - p_c}{P_{exp.} - p_{exp.}} \right) \left(\frac{P_{exp.} - p_{exp.}}{P_o} \right) \right]$$

or

$$K_{exp.} = K_c \left[\frac{273}{T_{exp.}} \right] \left[\frac{P_c - p_c}{P_o} \right]$$

and

$$x_{O_2} = h \left[K_c \left(\frac{273}{T_{exp.}} \right) \left(\frac{P_c - p_c}{P_o} \right) \right] \qquad (34)$$

which is the complete equation to be used with the Münzer and Neumann method of calibration.

Details of a calibration (and a test of the constancy of K_c):

(a) Determination of K_c: room temperature; 25°C.; manometer liquid, isocaproic acid; large size (ca. 60 ml.) Barcroft type flasks; 36 ml. water in each flask; P, 739.0 mm. Hg, 25°C., brass scale barometer.

T °C.	hc mm.	x_c µl.	K_c
10	28.8	81	2.72
15	45.2	122	2.70
20	68.3	186	2.71
25	34.8	95	2.73

These data are averages of 3-4 determinations made at each temperature.

(b) Calculation of x_{O_2}: room temperature, 25°C.; bath temperature, 25°C; P, 735.5 mm. Hg (corrected).

$$K_{O_2} = 2.73 \left(\frac{273}{298} \right) \left(\frac{735 - 23.7}{760} \right)$$

$$K_{O_2} = 2.34$$

$$x_{O_2} = h(2.34)$$

APPLICATIONS OF THE DIFFERENTIAL RESPIROMETER

RESPIRATION

The differential respirometer may be used in the same manner as the Warburg respirometer in determining oxygen uptake by the "direct method", i.e., alkali in the center wells, with or without liquid in the sidearms, of the flasks; K_{O_2} is obtained by calculation using the simplified equation, or the Münzer and Neumann method.

The differential respirometer may also be used to determine respiration by the "indirect method" of Warburg (see Chapter 4). Two respirometers are required. Unequal volumes of liquid and unequal volumes of gas, unequal volumes of liquid and equal volumes of gas, or equal volumes of liquid unequal volumes of gas may be used in the reaction vessels of the two respirometers. K_{O_2} and K_{CO_2} may be obtained as described above, and x_{O_2} and x_{CO_2} obtained by use of the equations developed for use with the indirect method.

In general, the differential respirometer can be used in any situation to which the Warburg respirometer is applicable; it can be used in those cases where it is desirable to increase the pressure of the gas in the reaction vessel above the pressure of the atmosphere.

The differential manometer has frequently been used in the past in attempts to measure the "differential effect" of some treatment. For example, tissue would be added to both flasks, but glucose to only one. The use of the differential manometer in this manner is not desirable. Frequently it robs the investigator of just that data which may be of vital importance, i.e., what does the tissue do without treatment?

FERMENTATION

The differential respirometer is used without alkali in the center wells (if the flasks possess these) and with or without liquids in the sidearms of the flasks. K_{CO_2} is obtained by using the simplified equation (i.e., Number 32).

PHOTOSYNTHESIS

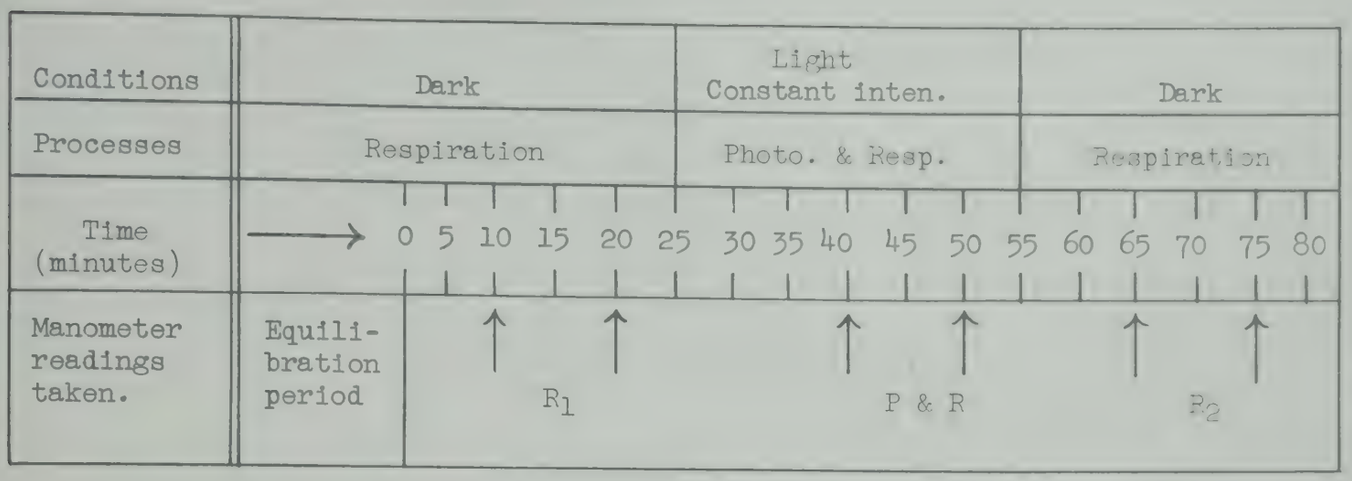
The discussion in this section is based on a consideration of photosynthesis as: carbon dioxide uptake and oxygen production by green cells in light.

In measuring photosynthesis the experiment usually involves three separate determinations of gas exchange; (1) a determination of respiration during a dark period, (2) a determination of gas exchange during the period the cells are illuminated, (3) a determination of respiration following the light period. These determinations are carried out in the order given above. It is readily understood that the gas exchange between the cell and its environment during the illumination period results from both photosynthesis and respiration, i.e., the observed rate of photosynthesis is less than the true rate because of the evolution of carbon dioxide and the uptake of oxygen by respiration which continues during the time the cells are illuminated. There is no way of determining respiration alone for illuminated green cells under experimental conditions where carbon dioxide is present, and it is known that the metabolism of Chlorella cells is not the same in the absence and in the presence of carbon dioxide (Emerson, Stauffer and Umbreit, 1944). Hence, respiration is measured immediately before and after the illumination period and the average value of these two determinations added to the observed rate of photosynthesis to give the true rate of photosynthesis.

As a matter of fact, in experiments seeking to establish the relationship between light absorbed and oxygen produced (CO_2 assimilated) the suspension of green cells is oftentimes so dense that, even though all of the light is absorbed, what one actually measures is a decrease in the rate of respiration during the time the cells are illuminated (c.f., Warburg and Negelein, 1922).

The following discussion will serve to distinguish two variations of the above described method of obtaining the true rate of photosynthesis. Both have proved satisfactory in determining the quantum efficiency of photosynthesis using the alga Chlorella.

A. "Steady state of gas exchange" method: The following diagram indicates the general relationships:



The duration of the light and dark periods may be varied, although 30-60 minute periods appear to be satisfactory. The R_1 and R_2 periods, which may differ in duration, are the same as or some proportion of the P & R period.

In this method, time is allowed for attainment of equilibrium in the 'plant cell - suspending liquid - gas phase' system before R_1, R_2 and P & R are measured, i.e., these quantities are measured during the steady state.

It is apparent that the true rate of photosynthesis (as µl. O_2 produced per minute) is obtained as follows:

$$\text{Photo.} = \frac{{}^{O_2}{}_{P\ \&\ R}}{\text{Minutes}_{P\ \&\ R}} + \tfrac{1}{2}\left(\frac{{}^{O_2}{}_{R_1}}{\text{Minutes}_{R_1}} + \frac{{}^{O_2}{}_{R_2}}{\text{Minutes}_{R_2}} \right)$$

This calculation may be considerably simplified by substituting the h values obtained for the different periods in the following equation:

$$h_{\text{Photo.}} = -\left[\tfrac{1}{2}\left(\frac{h_{R_1}}{\text{Minutes}_{R_1}} + \frac{h_{R_2}}{\text{Minutes}_{R_2}} \right) - \frac{h_{P\ \&\ R}}{\text{Minutes}_{P\ \&\ R}} \right] \qquad (35)$$

where the negative sign before the bracket allows for the correct substitution of either negative or positive values of $h_{P\ \&\ R}$.

Then:

$$\dot{X}_{O_2\text{Photo.}} = K_{O_2}(h_{\text{Photo.}})$$

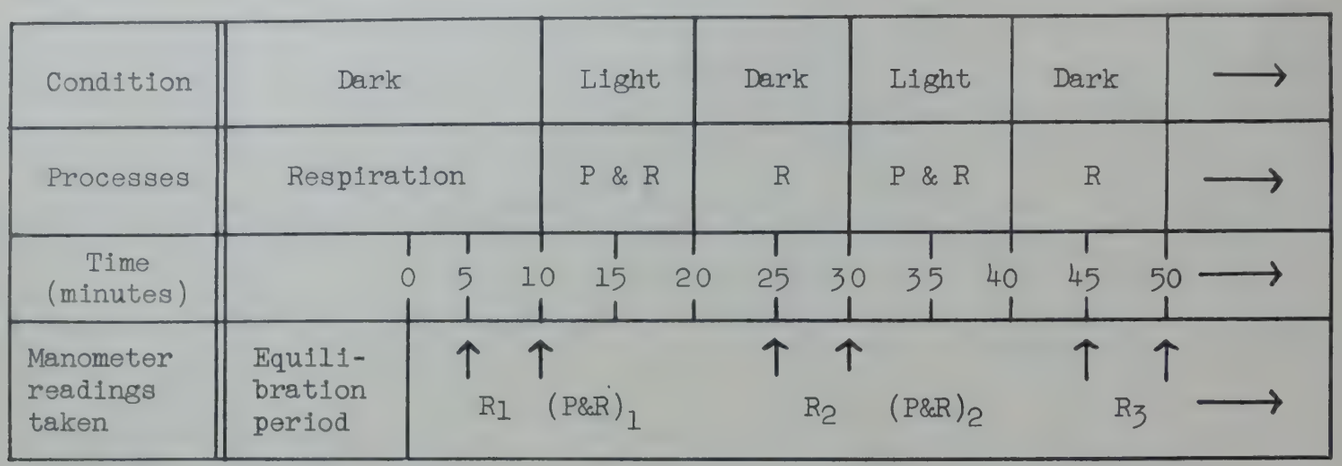
As an example: In a determination of photosynthesis in light of low intensity: h_{R_1}(10 minutes) = -4.0 mm.; h_{R_2}(10 minutes) = -3.8 mm.; $h_{P\ \&\ R}$(10 minutes) = -2.0 mm. (i.e., during the illumination period the rate of respiration was greater than the rate of photosynthesis).

$$h_{\text{Photo.}} = -\left[\tfrac{1}{2}\left((-0.4) + (-0.38) \right) - (-0.2) \right] = 0.19 \text{ mm.}$$

In another case, where high light intensity was used: h_{R_1}(10 minutes) = -2.0 mm.; h_{R_2}(10 minutes) = -2.1 mm.; $h_{P\ \&\ R}$(10 minutes) = + 4.0 mm.

$$h_{\text{Photo.}} = -\left[\tfrac{1}{2}\left((-0.2) + (-0.21) \right) - (-0.4) \right] = 0.605 \text{ mm.}$$

B. "Alternating light-and-dark periods" method: The following diagram indicates the general relationships:



As indicated, the rate of respiration is measured during the second 5 minutes of each dark period. The first 5 minutes allows for the attainment of equilibrum conditions of gas exchange for respiration after a period of combined photosynthesis and respiration. On the other hand, while photosynthesis begins as soon as the plant cells receive light and ceases when the light is turned off, the last vestiges of oxygen produced in photosynthesis requires one or two minutes to reach the gas space, and hence it would not be included in the measured oxygen if a manometer reading were taken the instant the light was turned off. The same reasoning holds when the rate of photosynthesis is less than the rate of respiration. Therefore, a manometer reading is taken at the end of the first 5 minutes of darkness. Manometer readings may be continued to be taken in this order for succeeding 10-minute light and dark periods for as long a time as is practical.

The true rate of photosynthesis is calculated as follows: Since respiration has been measured for two 5-minute periods (immediately before and 5 minutes after the photosynthesis-respiration period), the average respiration for the two periods is taken to represent the respiration occurring during each 5-minute period of photosynthesis-respiration. During the 15-minute period of photosynthesis-respiration, photosynthesis occurred for 10 minutes and respiration occurred for 15 minutes. Hence the true rate of photosynthesis for the 10-minute light period is obtained as follows:

$$\text{Photo}_{10\text{ minutes}} = 3\left(\frac{{}^{O_2}R_1 + {}^{O_2}R_2}{2}\right) + {}^{O_2}(P\&R)_1$$

The result can be obtained more quickly by substituting the h values obtained for the different periods in the following equation.

$$h_{10\text{-min. Photo.}} = -\left[3\left(\frac{h_{R_1} + h_{R_2}}{2}\right) - h_{P\&R}\right] \qquad (36)$$

where the negative sign before the bracket allows for the correct substitution of either negative or positive values of $h_{P\&R}$. As an example: In a determination of photosynthesis in light of low intensity: $h_{R_2} = -5.1$ mm.; $h_{R_3} = -5.1$ mm.; $h_{P\&R_2} = -10.1$ mm.

$$h_{10\text{-min. Photo.}} = -\left[3\left(\frac{(-5.1) + (-5.1)}{2}\right) - (-10.1)\right] = 5.2 \text{ mm.}$$

From the above discussion of the methods of measuring photosynthesis by means of the differential manometer, it is evident that in the reaction vessel: (1) both carbon dioxide and oxygen are present in the gas space and are dissolved in the solution in which the plant cells are suspended; (2) during respiration carbon dioxide is added to and oxygen is removed from the gas and liquid phases; (3) during photosynthesis-respiration, if the rate of photosynthesis exceeds the rate of respiration, oxygen is added to and carbon dioxide removed from the gas and liquid phases; (4) if the rate of photosyn-

thesis does not exceed the rate of respiration during the photosynthesis-respiration period the situation is the same as for (2) above, the rate of exchange only is decreased. In other words, both the oxygen and carbon dioxide content of the gas and liquid phases change. If, during respiration the CO_2/O_2 ratio is 1.0 the net difference in terms of total gas in the reaction vessel is zero; the same is true when the photosynthesis ratio, O_2/CO_2 is 1.0. There would be no change in the manometer reading were it not for the fact that α for oxygen is much less than the α for carbon dioxide. When oxygen is utilized in respiration V_g decreases due to the greater solubility of the carbon dioxide produced in the liquid present. By the same reasoning, V_g increases during photosynthesis.

.In extending the theory of the differential respirometer to include this situation: (1) K of the gas space must be determined, since there is no way of determining what proportion of its volume is occupied by oxygen and by carbon dioxide; (2) a means must be found to determine K_{O_2}, i.e., when we know what effect the addition of x amount of carbon dioxide and the removal of the same quantity of oxygen (or the reverse of this exchange) will have on h; (3) how a correction can be made when the photosynthesis ratio differs from 1.0. It does not matter what the respiratory ratio is if photosynthesis is calculated on the basis of the h values for the different periods (see equations 35, 36 above) for what occurs in the reaction vessel is essentially a change in h due to photosynthesis, and this is the only change measured.

(1) Determination of $K_{gas\ space}$: The K desired is the change in manometer reading, h, produced by adding x quantity of gas to the gas space when no gas is absorbed by the liquid present. It is assumed that the volume of liquid occupying V_f(and V'_f) is to be present during an experiment.

The K_{gs} may be determined by using a modification of the simplified equation. $V_f \alpha$ in the third term of the equation, which accounts for the quantity of the gas introduced that dissolves in the liquid, is omitted and the equation used in this form:

$$K_{gs} = h\left[\left(1 + \frac{A\ P_o}{2\ V'_g}\right)\left(\frac{V_g \frac{273}{T}}{P_o} + \frac{A\ 273}{2\ T}\right)\right] \qquad (37)$$

where the product of the terms within the brackets is the "gas space constant" of the respirometer. In reality this equation is a variant of the equation derived by Barcroft (1908). The original equation is:

$$K_{gs} = h\left(\frac{273}{T}\right)\left(\frac{V_g\ AP}{P_o}\right) \qquad (38)$$

and was used by Warburg and Negelein (1922) in deriving the constant of their differential manometer. Equation (37) has the advantage of allowing for different volumes of V_g and V'_g. The substitution of experimental values into equations (37) and (38) will yield the same results (within 2%) only if the cross-sectional area of the manometer capillary is less than 0.25 sq. mm.

The constant of the gas space may also be obtained by the Münzer and Neumann method. The general procedure is the same as that described in the section on calibration (p. 59), with the exception that a volume of glass beads equal to the volume of solution which will be used during an experiment is added to each vessel of the respirometer and calcium chloride drying tubes are attached to the end of the manifold and to the gas vents of the respirometer. The use of the calcium chloride drying tubes obviates any correction for the vapor pressure of the water vapor in the air of the room. It is not necessary that V_g and V'_g be adjusted to the same volume. However, the approximate volumes of the flasks can be determined by running in water from a burette, and the difference between V_g and V'_g in microliters of glass beads added to the larger flask, the compensation vessel. The constant is obtained from the h values observed when x volumes of dry gas is added to or withdrawn from the reaction-vessel side of the respirometer,

$$K_{gs} = \frac{x}{h}\left(\frac{273}{T}\right)\left(\frac{P_c}{P_o}\right) \qquad (39)$$

If dry gas is not used, a correction for the vapor pressure of water should be applied. In such a case P_c in the above equation is decreased by an amount p_c, which is the vapor pressure of water in the atmosphere of the room in which the calibration is made. If the air is 50% saturated with water vapor at 25°C., this correction reduces K_{gs} by approximately 1.5%.

The complete equation for the Münzer and Neumann method (Eq. 34) can be used to obtain this constant for experimental conditions different from those of calibration.

(2) Determination of K_{O_2} when equal amounts of carbon dioxide and oxygen are exchanged: The extension of the theory to determine K_{O_2} based on the difference in solubilities of carbon dioxide and oxygen in the liquids in the vessels has been described by Warburg and Negelein (1922). It is as follows:

Let: P_o = normal pressure in mm. of manometric fluid.

V_f = µl. of liquid in reaction vessel (also in V').

Y_{O_2} = µl. of oxygen released or absorbed.

Y_{CO_2} = µl. of carbon dioxide released or absorbed.

h = manometer reading, in mm.

α_{O_2} = absorption coeff. of oxygen at T.

α_{CO_2} = absorption coeff. of carbon dioxide at T.

V = change in volume of the gas space in the reaction side of the respirometer.

BV = µl. of oxygen added to or removed from the gas space.

$(B - 1)V$ = µl. of carbon dioxide added to or removed from the gas space.

hV = change in the partial pressure of oxygen when the volume of the gas space changes by an amount V.

$(h - 1)V$ = change in the partial pressure of carbon dioxide when the volume of the gas space changes by an amount V.

Then, $V = BV - (B - 1)V$

and $h = Bh - (B - 1)h$

Thus, $$Y_{O_2} = BV + \frac{Bh(V_f \alpha_{O_2})}{P_o} \qquad (40)$$

and $$Y_{CO_2} = (B - 1)V + \frac{(B - 1)h(V_f \alpha_{CO_2})}{P_o} \qquad (41)$$

Since $Y_{CO_2} = Y_{O_2}$

on eliminating B and Y_{CO_2} after combining equations (40) and (41)

$$Y_{O_2} = \frac{\left(V + \frac{hV_f \alpha_{CO_2}}{P_o}\right)\left(V + \frac{hV_f \alpha_{O_2}}{P_o}\right)}{\frac{hV_f(\alpha_{CO_2} - \alpha_{O_2})}{P_o}} \qquad (42)$$

In this equation, h represents the increase in pressure, ΔP, on the reaction side of the respirometer. Actually h is less than ΔP because of the compensatory effect of the increase in pressure on the compensation-vessel side. A correction may be applied by substituting ΔP for h, where

$$\Delta P = h + P\left(\frac{V_g}{V_g - \frac{Ah}{2}} - 1\right)$$

This correction reduces the value of Y_{O_2} by about 1%.

Since V is equal to hK_{gs},

$$X_{O_2} = h\left[\frac{\left(K_{gs} + \frac{Vf\alpha CO_2}{P_o}\right)\left(K_{gs} + \frac{Vf\alpha O_2}{P_o}\right)}{\frac{Vf(\alpha CO_2 - \alpha O_2)}{P_o}}\right] \qquad (43)$$

and K_{O_2} is equal to the quantity represented by the bracketed terms in the above equation.
(3) Correcting K_{O_2} when the photosynthetic ratio, O_2/CO_2, is not unity:

Let $\frac{Y_{O_2}}{Y_{CO_2}} = Z$

then equation 42 takes this form,

$$X_{O_2} = h\left[\frac{\left(ZK_{gs} + Z\frac{Vf\alpha CO_2}{P_o}\right)\left(K_{gs} + \frac{Vf\alpha O_2}{P_o}\right)}{K_{gs}(Z - 1) + Z\left(\frac{Vf\alpha CO_2}{P_o}\right) - \frac{Vf\alpha O_2}{P_o}}\right] \qquad (44)$$

The above equation is the complete equation to be used with the differential manometer in determining photosynthesis provided the photosynthesis ratio is known. It so happens, however, that a determination of the photosynthesis ratio is not an easy matter. Many workers have either assumed it to be 1.0, or they have used the value obtained by Warburg and Negelein (1922). As a matter of fact, Warburg and Negelein did not determine this ratio under conditions strictly comparable to those which prevailed during their measurement of the quantum efficiency of photosynthesis. They used a glass vessel filled with a gas mixture and a suspension of algal cells, from which samples of gas were withdrawn from time to time and analyzed for oxygen and carbon dioxide. They obtained an average value of 1.1 in three experiments.

It is theoretically possible, but practically impossible to determine the photosynthesis ratio under ideal conditions using the differential respirometer. It would be possible to determine it by the indirect method of Warburg (see Chapter 4), in which the reaction vessels of two respirometers would contain the same volume of cell suspension (and concentration of cells) but with unequal gas spaces; the cell suspensions being exposed to exactly the same temperature and light intensity at the same time. It is doubtful whether any laboratory possesses such a set-up. The nearest approach is to carry out two successive experiments using equi-volume samples of the same cell suspension in flasks of unequal gas volume exposed at the same temperature to virtually identical quantities of light (c.f., Emerson and Lewis, 1941). As indicated above, X_{O_2} and X_{CO_2} can then be obtained by using the indirect method of Warburg, which can be applied directly to the differential respirometer when K_{O_2} and K_{CO_2} are known.

Because of the difficulty of obtaining the photosynthesis ratio (some investigators observe a "burst of CO_2" when algal cells, for instance, are illuminated) and because it effects a simplification of the whole procedure of determining photosynthesis, many workers favor the use of carbonate-bicarbonate CO_2-buffer solutions. Without going into the question of the effect of such solutions on the metabolism of the cell (Warburg, O., 1919; Manning, et al., 1938; Emerson and Lewis, 1942; Pratt, 1943), it is readily understood that if the partial pressure of carbon dioxide in the gas space of the reaction vessel of the respirometer is maintained constant, the change in h is due solely to the oxygen added to or removed from the system by the plant cells. Thus, one need only determine the K_{O_2} for the respirometer containing the particular CO_2-buffer under the conditions of experimentation. There are certain precautions, however, to be borne in mind. In particular: the carbonate-bicarbonate CO_2-buffers change as carbon dioxide is added to or removed from solution; this in turn changes the partial pressure of the carbon dioxide in the gas space of the respirometer vessel. Warburg (1919) has called attention to this

fact, and has indicated the working range in terms of the quantity of carbon dioxide that can be removed from or added to the solution without introducing an error of more than 1% in the h values obtained. He also pointed out that the higher the pH of a carbonate-bicarbonate solution the shorter the period of time the plant cells, i.e., Chlorella can remain in it without showing a decrease in photosynthetic capacity. Pratt (1943) has considered this question of the physiological effect of sodium and potassium bicarbonates on the rate of respiration and photosynthesis of Chlorella vulgaris. As a result of his study, he recommends a solution consisting of 0.035 M $KHCO_3$ and 0.065 M $NaHCO_3$. He found that in such a solution the accelerating and depressing actions of potassium and sodium salts, respectively, were balanced and the initial rate of photosynthesis was maintained virtually unchanged for fifteen hours.

Table XIV contains some of the pertinent data for various solutions of sodium carbonate-bicarbonate CO_2-buffers. Solutions of this type were used by Warburg (1919) in determining the effect of carbon dioxide concentration on the rate of photosynthesis in Chlorella.

TABLE XIV

Carbonate-bicarbonate Mixtures

Mixture No.	Composition, mls. Na_2CO_3 0.1 M	$NaHCO_3$ 0.1 M	Na millimoles Liter	CO_2 Moles/L 25°C.	pH* 25° C.
1	85	15	185	0.53×10^{-6}	10.42
2	80	20	180	1.0 "	10.30
3	75	25	175	1.7 "	10.19
4	70	30	170	2.6 "	10.10
5	60	40	160	5.3 "	9.93
6	50	50	150	9.8 "	9.79
7	35	65	135	2.3×10^{-5}	9.51
8	25	75	125	4.3 "	9.32
9	15	85	115	9.1 "	9.08
10	10	90	110	15.0 "	8.91
11	5	95	105	33.0 "	8.69

*Routine determinations made with a glass electrode.

The data in the above table are presented only to show the composition of some CO_2-buffers that have been used, and to point out the relatively high pH values of such solutions. In case one desires to use such buffers, it is imperative that their effect on the particular process under investigation be determined. It certainly appears that the recommendations of Pratt (1943) should be considered.

Data of an Experiment: The differential respirometer was set up with 36 ml. of algal cell suspension in the reaction vessel (left) and 36 ml. of the nutrient solution without the algal cells in the compensation vessel. Before beginning the actual experiment, the vessels were flushed with 5%-CO_2-air mixture for one hour in order to saturate the liquids with carbon dioxide at this partial pressure. The algal cells were irradiated with red light of low intensity. The shaking of the flasks was stopped momentarily when a reading of the heights of the manometer liquid in the limbs of the manometer was made.

The following data were taken:

TABLE XV

Data of a Typical Experiment in Photosynthesis

Period	Time min.	Environment	Manometer Readings, in mm. Left	Right	Difference	Increment
---	0	Light	146.7	146.9	0.2	
	5	"				
	10	"				
	10	Dark				
	15	"				
R_1			147.5	145.9	-1.6	
	15	Dark	147.5	145.9	-1.6	
	20	"	148.0	145.4	-2.6	
$(P\&R)_1$						-1.0
	20	Light	148.0	145.4	-2.6	
	25	"				
	30	"				
	30	Dark				
	35	"				
R_2			149.4	144.0	-5.4	-2.8
	35	Dark	149.4	144.0	-5.4	
	40	"	149.9	143.4	-6.5	
$(P\&R)_2$						-1.1
	40	Light	149.9	143.4	-6.5	
	45	"				
	50	"				
	50	Dark				
	55	"				
R_3			151.0	142.0	-9.0	-2.5
	55	Dark	151.0	142.0	-9.0	
	60	"	151.5	141.6	-9.9	
$(P\&R)_3$						-0.9
	60	Light	151.5	141.6	-9.9	
	65	"				
	70	"				
	70	Dark				
	75	"				
R_4			152.6	140.4	-12.2	-2.3
	75	Dark	152.6	140.4	-12.2	
	80	"	153.0	139.9	-13.1	-0.9

The amount of photosynthesis during the $(P\&R)_3$ period, for instance, can be calculated by the use of equation (36) above. It is:

$$h_{10\text{ min. Photo.}} = -\left[3\left(\frac{(-.9)+(-.9)}{2}\right)-(-2.3)\right] = 0.5 \text{ mm.}$$

This value multiplied by the constant of the manometer is the quantity of oxygen liberated during the 10 minute exposure to light. As is often the case, and as represented above, changes in light intensity from one P&R period to the next result in different values of photosynthesis for the different periods ($(P\&R)_1$, $(P\&R)_2$ etc.).

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Chapter Eight

METHODS OF PREPARATION AND STUDY OF TISSUES

INTRODUCTION

The manometric methods described are accurate and capable of measuring certain reactions with speed and precision. When applied to living tissues or preparations therefrom they are capable of measuring the rate and the course of many important processes. One must obtain, however, tissues or preparations in which the reactions one wishes to study occur. Since these reactions are carried out by enzymes and are influenced by an array of physical and physiological conditions, the material employed in the manometric estimations must contain the components of the reaction to be studied.

That some of the multitude of interrelated chemical reactions which occur in vivo could be obtained in vitro has permitted the advances which have occurred in our knowledge of respiration and metabolism. The problem which faces every investigator is the development and study of the means by which reactions occurring in intact tissues may be separated and studied. The techniques applicable to one type of tissue are not necessarily suitable for another. It is, in fact, the knowledge of the physiology of the tissue with which one is working which permits one to select a technique of preparation which will be suitable for the tissue one is employing and the reaction or process one wishes to measure. We wish to emphasize that the techniques described below are not mutually exclusive nor are they all equally applicable. The question of which method to apply (if any) of the techniques listed is the responsibility of the individual investigator just as he assumes responsibility for the interpretation of the results. Certainly some of the techniques described below are useful but modifications of these or even the development of entirely new ones may be necessary to approach specific problems.

The subjects considered in this Chapter are divided into three sections dealing with Animal, Plant and Microbial Tissues.

METHODS OF PREPARING ANIMAL TISSUES

P. P. Cohen

TISSUE SLICE TECHNIQUE - INTRODUCTION

The tissue slice technique was developed in most of its details by Warburg and his co-workers in their studies of tumor metabolism (Warburg, 1926). By the use of tissue slices, the more complicated and uncontrollable aspects of whole organ or organism metabolism are minimized on the one hand, and the less certain effects of mincing, homogenizing or extracting are excluded on the other hand. In a word, the tissue slice is thought to represent organized surviving tissue, the metabolism of which qualitatively, if not quantitatively, reflects that of the original tissue. Further, the tissue slice technique allows for controlled variations in the suspending medium in addition to chemical analysis of the latter for changes in metabolite content. An excellent critical analysis of the tissue slice method in manometric experiments has recently been published by Laser (1942).

PREPARATION OF SLICES OF ANIMAL TISSUES

It is usually possible with practice to slice most animal organs free hand with either a straight edge or a safety razor. However, where the size of the organ is very small, as for example a small tumor nodule, a mouse kidney, etc., or where the organ lacks consistency, such as brain, the free hand method of slicing even by the expert leaves much to be desired. A simple and most effective improvement in the technique of tissue slicing applicable to all organs and tissues is that introduced by Deutsch (1936). The principle of this technique is as follows:

The piece of tissue to be sliced is held firmly between two pieces of frosted glass and the tissue sliced by means of a razor blade, the latter being guided by the top frosted glass. In practice it is soon appreciated that the pressure necessary to keep the tissue fixed while slicing varies from tissue to tissue, and further that the thickness of the slice can be estimated by its translucency through the frosted glass.

In the writer's hands the method of Deutsch has proved to be applicable to a wide variety of tissues. The following equipment and procedure has proved most satisfactory.

Equipment:

1) A piece of frosted glass, approximately 5 cm. square, mounted by means of paraffin on a heavy base, 5-6 cm. in height (an inverted cold cream jar has been found very satisfactory).
2) Frosted microscope slides; these are readily prepared from ordinary glass slides by rubbing with emery powder.
3) Razor blades. The three-holed variety broken in half (length-wise) are satisfactory.
4) Razor blade holder. This is conveniently made from a piece of brass approximately 10 x 8 x 2 mm. with a hole drilled through one end holding a brass bolt and nut. The nut should have a diameter of about 8 mm. so as to provide a good purchase on the razor blade. The broken blade is placed so that the bolt fits into one of the end holes. The blade is fixed by tightening the bolt with a screw driver.

Procedure:

The procedure will vary somewhat from tissue to tissue. However, in general one proceeds as follows:

A piece of hard filter paper, approximately 2 cm. square, is placed over one corner of the frosted glass and moistened with saline. The piece of tissue to be sliced (usually about 1 cm. in diameter) is placed on the filter paper. The frosted slide is then dipped in saline and applied to the top of the piece of tissue with gentle pressure and held in place with one hand. The razor blade is then moistened with saline and by means of the holder the blade is closely applied to the under surface of the frosted microscope slide. With experience it will be found possible to adjust the pressure on the tissue with the one hand and effectively slice with the other. The thickness and uniformity of the slice can be readily appreciated by the appearance of the slice through the microscope slide. (By cutting across the corner of the frosted-glass base the operator's hand, and the razor

blade and holder, will be free of obstruction). The slice is then transferred to a petri dish containing a suitable saline mixture. The slices should be handled by means of a pair of fine but blunt-ended forceps. When transferring the slices to the Warburg flasks, the slices are gently dried by touching with a piece of hard filter paper.

ESTIMATION OF THE THICKNESS OF TISSUE SLICES

Since the rate of diffusion of gases and metabolites will be determined in part by the thickness of the tissue slices, it is essential that they be of uniform thickness within certain limits. These limits are determined by the diffusion constants of the reacting substances, the rate of metabolism of the tissue in question, etc. As derived by Warburg (1930), the limiting thickness in cm., d', for O_2 consumption of slices of a given tissue can be calculated from the equation

$$d' = \sqrt{8\, C_o\, \frac{D}{A}}$$

where D = the diffusion constant for O_2 in ml. (N.T.P.). According to Krogh, the rate of diffusion of O_2 at 38° through a tissue of 1 cm.^{2} cross section is 1.4×10^{-5} ml. per min.

A = the rate of respiration $\left(\frac{\text{ml. } O_2 \text{ uptake}}{\text{ml. tissue x min.}} \right)$

C_o = the O_2 concentration outside the slice (in atmospheres).

Taking 5×10^{-2} as the value for A for liver slices, 1.4×10^{-5} for D, and 1.0 and 0.2 for C_o for pure O_2 and air respectively, d' is calculated to be 4.7×10^{-2} cm. for pure O_2 and 2.1×10^{-2} cm. for air. In other words, if the gas phase is air, liver slices no thicker than 0.2 mm. can be used. Slices of this thickness are not only difficult to prepare but also are very fragile and consequently very difficult to work with. On the other hand, slices 0.3 mm. in thickness can be cut with little difficulty and can be handled and shaken without danger of damage. However, even with slices of this thickness the gas phase must be pure O_2. Under these conditions the O_2 tension at the center of the slice will be about 0.6 atmospheres. Similar calculations have been carried out for CO_2 by Warburg (1930).

In practice it is quite easy to estimate the thickness of a given slice by its translucency and by the manner in which it curls up on itself when held up by a pair of fine forceps. However, in order to establish the correct thickness in terms of the above visual criteria it is best to measure a few selected slices of the different tissues. This is most readily done by placing the slices in a petri dish of Ringer's solution under which is placed a piece of squared millimeter paper. The slices are trimmed to a rectangular shape and their areas measured by counting the squares covered. The volumes of the individual slices are then calculated from their wet weight. The thickness of a given slice is then obtained by dividing the volume by the surface area.

TEMPERATURE OF TISSUE AND MEDIUM

In the author's experience it has been found desirable, and in some instances essential, to keep the tissue and medium at low temperatures while the slices are being cut. This is readily accomplished by keeping the petri dish containing saline and slices on cracked ice, and by filling the cold cream jar with cracked ice and placing it in a petri dish. The latter insures the tissue being kept cold while it is being sliced. The medium to be used for suspending the slices is cooled by storage in the refrigerator up to the time of use. The organs or tissues when removed from the animal are placed on cracked ice directly to chill rapidly and are then placed in small beakers which are surrounded by cracked ice.

DRY WEIGHT OF TISSUE SLICES

Metabolic quotients are usually expressed in terms of mg. dry weight of tissues. As pointed out in Chapter I, this procedure may give rise to erroneous comparisons of metabolic rates of different tissues. Since the metabolic activity is associated more directly with the nitrogen (protein) content of a given tissue, it would seem desirable to include

nitrogen determination when comparing tissues. Experiments in which tissues from an animal on one diet are compared with those from an animal on another diet may show differences which are more apparent than real on the basis of dry weight. This is particularly true in the case of liver the composition of which is so markedly influenced by diet. Thus it is possible to demonstrate an apparent increase in the content of certain enzymes of liver by merely starving the animal. This can be shown to be due in part to the decrease in glycogen with a consequent increase in protein (enzyme) concentration. This precaution of evaluating metabolic quotients, such as Q_{O_2}, on the basis of dry weight in comparative experiments cannot be overemphasized.

Tissue slice dry weights may be obtained in one of two ways:

1. Weighing moist slices on a Torsion balance and then taking a sample to dryness to determine the wet weight/dry weight ratio.
2. Determining dry weights of the tissues in each flask at the end of the incubation without regard to wet weight.

With good slicing technique and care in handling the slices, reliable and consistent results are obtained with either method. The chief objection to the first method is that it is difficult to insure a uniform H_2O and saline content when transferring the slices from the saline to the balance. The question as to whether the one or the other method gives more nearly correct values is not possible to answer. With the first technique it is assumed that the added weight of tissue is maintained and is metabolically active throughout the experimental period. This is certainly not true for all tissues. In the second method, the tissue removed from the experimental flask does not include, usually, the fragments broken off during the course of shaking. It is assumed in this instance that these fragments are not contributing to the metabolism of the system. Whether or not this is actually the case it is not possible to say. However, it may in part explain why the Q_{O_2} values obtained with the second method tend to be somewhat higher than those obtained with the first.

For the purpose of determining dry weights of tissue slices, it is convenient to use containers of small weight to allow accurate weighing of a few milligrams. The author uses small flat bottomed vials measuring approximately 10 mm. in length and 8 mm. in diameter, and which weigh between 300-500 mgs. These vials are numbered and their weights recorded. Vials of this size are satisfactory for dry weights of 10-20 mgs.; larger vials are used where the quantity of tissue exceeds this amount. Small watch glasses, crucibles, etc. can also be used. The slices should be dried for about 2 hours at a temperature of 105-110°C.

MINCING OF ANIMAL TISSUES

The purpose in mincing tissues for metabolic study is to reduce them to particles of such size as to permit adequate perfusion in and out of the suspending medium, and also to provide a uniform tissue suspension of relatively high concentration. In comparison with the tissue slice, the mince particles contain a high per cent of damaged cells. The chief use of the tissue mince in recent times has been in the study of muscle metabolism, and in particular, pigeon breast muscle. Several mechanical devices for reducing tissues to a uniform mince have been described and successfully employed in metabolism experiments. A few of these will be discussed, Latapie Mincer. (See Fig. 20).

The feature of this instrument is that it is possible to control not only the rate of turning of the cutting discs but also the rate at which the tissue is forced against these discs. It is thus possible to obtain minces of different degrees of fineness, by varying the rates of turning of the two cranks, (See Fig. 20). In most metabolic experiments it is desirable to have a mince which can be pipetted after it is suspended in the proper medium. This permits more rapid manipulation and uniformity of tissue content from flask to flask. On the other hand, too fine a mince is usually avoided in order to maintain some degree of integrity of the cells making up the particles. An additional feature of the Latapie is that sterile conditions may be maintained if desired.

The popular Latapie Mincer is intended for use with relatively large amounts of tissue, that is, of the order of 25 grams or more. It is thus particularly suitable for a tissue like pigeon heart muscle. However, where small animal tissues are to be employed

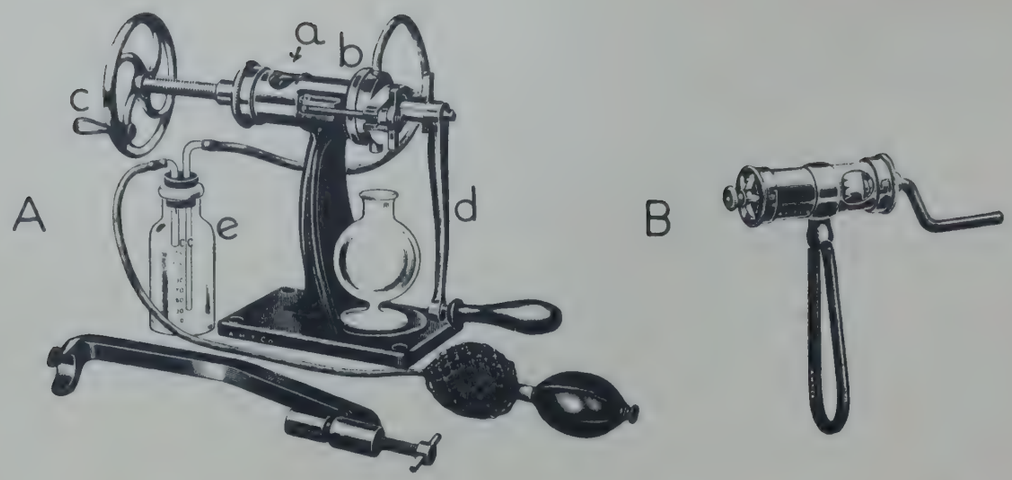

Fig. 20. Tissue Mincing Mills

A. The Latapie Mill. The material to be ground is fed into opening "a" and is gradually forced against the cutting discs (at "b") by turning wheel "c". The material is ground by turning the crank "d" while the remainder of the apparatus is designed to supply fluid if necessary "e".

B. The Apolant Mill. (Both mills are obtainable from the Arthur H. Thomas Company).

the instrument is too large. A smaller model is available for this purpose (Arthur H. Thomas Co., Philadelphia, Pennsylvania).

Apolant and Seevers and Shideman Mincers:

Other types of small mincing apparatus have also been used. Thus the Apolant type (see Fig. 20) is available on the market and is suitable for grinding tissues weighing in the neighborhood of 1 gram. In the author's experience, this type of mincer gives a low yield of properly minced tissue and thus is of little use where the quantity of tissue is limited. More recently a new type of micro-mincer has been reported by Seevers and Shideman (1941) which appears to be ideally suited for use with small amounts of tissue since it is adaptable to varying quantities of material. Thus the authors report that amounts of tissue from 0.25 to 30 grams can be conveniently minced. The yield of mince from 250 milligrams of tissue is reported as 200 milligrams. In common with the Latapie Mincer principle, the tissue cannot be forced through without being cut since the cutting blades are synchronized to turn as the plunger advances. This feature guarantees a uniform particle size of tissue. A comparison of the respiration of homogenized liver and liver minced by this apparatus has been reported by Potter (1941).

Fig. 21. The Waring Blendor

Waring Blendor:

Where relatively large amounts of tissue are available and a fine mince, of the consistency of a homogenate, is desired, the Waring Blendor is recommended. This instrument is now made with a small size glass container to handle 50-100 ml., and is obtainable from the Central Scientific Co., Chicago, Ill. (see Fig. 21).

HOMOGENATES

Homogenates consist of suspensions of the particulate components of protoplasm in a solution of the freely soluble components. They are prepared by grinding the tissues to such a degree that the cell structure is destroyed.

In essence they are cell-free preparations. Description of their preparation and use is given in Chapter 9.

SPECIAL TISSUE PREPARATIONS

Muscle:

Skeletal muscle does not yield satisfactory tissue slices chiefly for the reason that the muscle cells are relatively large. As a result, slices have a large percentage of damaged cells per slice. Two preparations of skeletal muscle which have been used successfully are fiber bundles described by Richardson et al. (1930), and young rat diaphragm. The former has been prepared particularly from dog muscle by careful dissection, and the technique was found to yield a uniform preparation of intact fibers. Rat diaphragm is a very convenient muscle preparation since it involves a minimum of preparation. The rats should preferably weigh in the neighborhood of 100 grams. In this weight range, the diaphragm will have a thickness of about 0.3 mm.

Smooth muscle preparations have the disadvantage of contracting to thick pieces of tissue and consequently may not be suitable because of diffusion difficulties. A convenient and little used source of smooth muscle for slicing is bird gizzard. The author has found pigeon gizzard very suitable for slicing since it yields very thin slices which do not contract appreciably. It is further convenient in that a single gizzard will yield enough slices for the most elaborate metabolic experiment. Other convenient smooth muscle sources are:

1. The inner circular layer of the small intestine, which may be dissected free from mucosa and outer layers, and
2. The medial layer of middle-sized arteries from larger animals. The advantage of the latter source of mammalian smooth muscle over that of many others is that one may obtain relatively pure preparations of convenient thickness and amount.

Testis:

The preparation of testis suitable for metabolic experiments is best accomplished by "teasing out" the tissue. The outer capsule is cut with a scissors and the testicular tissue is expressed into saline by manual pressure. The seminiferous tubules are then teased apart by combing the tissue with two pairs of blunt end, curved forceps. This technique insures practically intact tubules.

Retina:

This structure can be used directly in metabolic experiments by merely stripping the sectioned eye ball. Sheep and pig retinae are particularly useful since they represent easily obtainable material. Rat retinae may be used but, of course, a large number of eyes are required to yield a sufficient amount of tissue. Extreme care must be exercised in handling this delicate tissue particularly if anaerobic experiments are to be performed. In the absence of substrate retina may lose a large share of its metabolic activity if kept anaerobic for as little as 2-3 minutes.

Nuclei:

The isolation of cell nuclei suitable for enzymatic study has been reported by Dounce (1943). The technique involves mincing the tissue (liver) in the presence of citric acid. The enzymatic content of these preparations has been reported by Dounce and also by Lan (1943).

METHODS OF PREPARING PLANT TISSUES

J. F. Stauffer

HIGHER PLANTS

In general, the higher plant does not possess organs constituted of massive tissues as does an animal. The most actively respiring regions of a plant are those where growth, in the sense of increase in number of cells, is progressing most rapidly, namely the meristem regions of the stem and root, the developing flower and fruit, the germinating seed, and seedling. As a rule every plant cell progressively develops from the meristematic to the mature state. The rate of respiration also decreases as the cell matures; too, as the organ of the plant matures, cells are transformed into dead xylem elements, for example, which further lowers the respiratory rate of the organ. Furthermore, the respiratory rate of the higher plants is considerably less than that of most animal tissues.

Since the course of many experiments on respiration are dictated by the very practical consideration of using enough tissue to be able to measure respiration over a relatively short period of time, the introduction of the manometric methods requiring relatively small quantities of plant tissue has allowed considerably more latitude in the choice of material and type of experiments.

We do not propose to discuss at length the preparation of all the various plant parts for use in the study of the several aspects of respiration. Rather, the discussion will be limited to a few general cases together with indications as to where other accounts can be found.

Storage organs of a number of different types of plants have been used extensively. Potato tubers and carrot roots are good examples. Both have a high food content and contain a relatively high percentage of uniform, parenchymatous cells 80-150 microns in width. In the case of the potato tuber, plugs of tissue are removed from the interior by means of a cork borer having a diameter of 6-12 mm. These plugs are then worked up into slices, 0.4-0.75 mm. thick with a hand microtome. In some cases a series of razor blades separated by washers has been used with considerable success to speed up the slicing. The slices are then washed for 12-24 hours in running tap water, rinsed several times in distilled water, blotted with filter paper and transferred to the solution contained in the manometer vessel. The washing serves to remove organic matter from the cut cells and also eliminates certain inequalities in respiration partially associated with the period succeeding cutting (Steward, 1932). Somewhat the same procedure was followed by Turner (1938) in preparing slices of carrot root. He recommends that the whole organ be sliced and then cylindrical discs be cut from the slices to minimize bruising of the tissue. Marsh and Goddard (1939) used carrot slices 6 mm. in diameter and 0.5 mm. thick suspended in pH 5.9 phosphate buffer in determining the effects of cyanide, azide and carbon monoxide on respiration.

Root tips and segments of roots have also been used. It is relatively easy to grow the plants in liquid culture until the root systems have developed sufficiently to yield the desired quantity of material. Tips of the roots of uniform length and diameter are removed, rinsed in distilled water or in the suspending solution, blotted and transferred to the solution in the vessel of the respirometer It is necessary to randomize the roots in the different samples to overcome inequalities in respiratory rate. This method works quite well when only the extreme tip of the root is used; presumably segments of roots having root hairs would not work as well owing to variable injury to the very fragile root hairs. Machlis (1944) has described in detail a method for producing barley roots 12-15 cm. long, relatively free of root hairs, and of a uniform diameter slightly under 0.5 mm. He also describes a method of preparing segments of the roots for use in a study of the effects of certain inhibitors and the four-carbon acids on respiration. Root tips from seedlings are also convenient. The seeds may be sterilized for 10-20 minutes in a 10% solution of freshly prepared and filtered calcium hypochlorite solution, washed thoroughly in sterile water, and, after soaking for several hours in sterile water, germinated at room temperature on moistened, sterile filter paper in petri dishes or large, covered crystallizing dishes. The root tips are excised, blotted lightly and transferred directly to the solution in the vessel of the respirometer (Henderson and Stauffer, 1944). This same technique of seed sterilization and germination was also used in obtaining excised roots tips of tomatoes for culture in a purely synthetic medium.

For further details regarding the culture of excised root tips and isolated plant tissues, see White (1943).

Extracts of plant tissues are easy to prepare and find a wide application in the study of the various respiratory phenomena. In many instances an extract may be prepared by grinding a representative sample of the whole plant, organ, or tissue to a pulp in a mortar. The addition of a small amount of sand may facilitate this operation. For larger samples, a food chopper equipped with a fine-toothed cutter or a blendor (Waring type) may be used to advantage. The more liquid portion of the macerated material can be separated from the debris by pressing it out by hand through muslin or 2-3 layers of fine cheese-cloth. The liquid may be further freed of suspended particles by centrifuging or by allowing it to stand 10-12 hours at 2-4°C.; this latter treatment appears to work well in obtaining clear extracts from chlorophyllous tissues. The extract may be used immediately, or mixed with a buffer solution, whichever is most suited to the experiment in question. A variant of the above procedure is to freeze the tissue at -8 to -20°C. overnight, thaw, pass through a food chopper with a fine cutter, and press out the sap through felt or cheese-cloth pads in a hydraulic press. Extracts obtained in this manner appear to be more representative of the tissue as a whole, and, at least at first, more active than when the freezing step is omitted. Bunting and James (1941) in a study of carboxylase and cocarboxylase in barley prepared extracts from seedlings frozen overnight at -12°C. by pressing out the sap through muslin by hand. They also tried pulping the seedlings before pressing out the sap, and found that this treatment gave an extract that was more active than that from the unpulped seedlings. However, the difference in activity of the two disappeared after a time. Sap extracted by hand from barley shoots following overnight freezing at -12°C. and thawing was used by James and Craig (1943) in investigating the ascorbic acid system as an agent in respiration. Albaum and Eichel (1943) prepared extracts of whole embryos of oats as follows: 50 embryos were dissected free of the endosperm, ground in sand in 2 ml. of M/15 phosphate buffer, centrifuged lightly and the supernatant liquid decanted and stored in an ice chest overnight. Berger and Avery (1943) ground oat coleoptiles with twice their weight of water, and filtered or centrifuged the extracts for use in determining dehydrogenase activity by means of the Thunburg technique (see Chapter 12). Albaum and Umbreit (1944) prepared extracts of embryos of germinating oats for use in determining phosphorus transformations (see Chapter 15) by homogenizing the material in a minimum amount of ice cold distilled water with a stainless steel homogenizer (see Chapter 9 for a description of this technique). It would appear that homogenates of plant tissue would be more active, in respect to respiratory activity in general, than extracts from pressed, or ground and pressed tissue because the cell debris on which some of the enzymes may be absorbed, and yet remain active, is not removed. It has been used successfully in the study of transamination in oat seedlings by Albaum and Cohen (1943). Whichever technique, or modification of a technique, one employs in preparing a plant extract in the study of a particular problem, it goes without saying that the effect of each treatment in the preparation of the extract should be examined as to its effect on the activity of the final preparation.

PREPARATION OF ALGAL CELL SUSPENSIONS

There are a number of unicellular green algae which may be grown in the laboratory under standardized conditions as a source of plant material for use in studies of photosynthesis and respiration. The suitability of such plants for photosynthetic studies has been outlined by Manning, et al. (1938). In many respects the same considerations hold true for their use in respiratory studies. At present a number of species of Chlorella are maintained in pure culture in several botanical laboratories. Without any intention of implying that Chlorella is the only alga recommended, but because of its wide use at present and because of our experience with it, a general method for its culture is outlined below. With slight modification of conditions such as the composition of nutrient solutions, salt concentration, light, temperature, etc., this method can be used for the production of large numbers of cells of many other algae. In fact the method has been used in culturing a species of Dactylococcus, and with relatively slight changes for growing the brine flagellate Dunaliella salina (Weybrew, 1942). The method probably had its inception during the course of the experiments of Warburg (1919) and Warburg and Negelein (1922) on photosynthesis. In its present form it closely follows the description given by Manning, et al. (1938).

It is necessary to have the alga in pure culture. Impure cultures can be diluted, plated out on agar medium in petri dishes, and an uncontaminated colony picked off and

propagated (see Bold, 1942, for further details). This is rather time consuming with a long period of waiting until a colony is produced from a single cell. If possible a little of a pure culture should be purchased or obtained from a laboratory where the organism is being maintained. To continue the stock, and as a source of inoculum for the liquid cultures to be described below, the alga can be satisfactorily grown on slants of agar-solidified nutrient medium (see below) in ordinary test tubes. Of course, one must exercise the usual precaution against contaminants; the usual bacteriological methods are applicable. After having built up a sufficient supply of stock cultures, these can be easily maintained by bi-monthly transfers of cells onto fresh slants. The cells multiply readily when exposed to natural or artificial light.

The algal cells to be used in preparing the suspensions are grown in liquid cultures. A loopful of cells from a stock culture is introduced into 100-150 ml. of sterile nutrient solution (see below) contained in a 250 ml. Erlenmeyer flask. The flask is provided with a rubber stopper containing two L-shaped glass tubes. One of the tubes extends almost to the bottom of the flask and serves to introduce air or gas mixtures below the surface of the solution. The bubbles of gas also agitate the solution and render constant shaking unnecessary. The other tube is short and serves as the outlet for the gas mixture. The external ends of both tubes are lightly plugged with cotton. When the assembled culture flasks are to be sterilized by autoclaving, a strip of paper is placed around the rubber stopper where it contacts the neck of the flask, the flasks are placed in a metal tray and a paper cover is fitted over the whole. The tray and contents are removed directly from the autoclave to a cabinet or room that has been recently steamed down and allowed to cool. The flasks are then inoculated with the algal cells. The paper strip is removed and the rubber stopper forced into the neck of the flask. Finally, hot paraffin-bee's wax mixture (4-1) is brushed over the glass-rubber joint to render it gas tight. When this procedure is followed, the flasks are seldom contaminated. The flasks are now placed on a wire rack submerged 4-6 cm. in a shallow glass-bottomed, constant temperature tank. The flasks are connected in series (2-15 flasks) by means of short pieces of rubber tubing. Air or a mixture of CO_2 in air (4-5%, prepared by partial pressure method, stored in a carboy or compressed in a cylinder) is constantly bubbled through the flasks at a rate such that the CO_2 content is uniform in all of the flasks of the series. The cultures are illuminated from below with ordinary incandescent or fluorescent bulbs of sufficient wattage to give the desired light intensity. It is not necessary to shake the flasks constantly; shaking them by hand once or twice daily is sufficient. In practice, cultures of the type described in a 24 x 36 inch tank, illuminated constantly with 6 200-watt unfrosted bulbs at a distance of about 3 feet, produce 100-150 $mm.^3$ of packed wet cells per flask in 5-6 days at 22°C. when aerated with 5% CO_2-air mixture.

The following media are given since we have found them to be satisfactory. For others, see Bold (1942).

Medium for agar-slant stock cultures		Medium for liquid cultures	
$NaNO_3$	0.25 gm.	KNO_3	2.53 gm.
KH_2PO_4	0.25 gm.	KH_2PO_4	2.72 gm.
$MgSO_4 \cdot 7H_2O$	0.25 gm.	$MgSO_4$	2.40 gm.
$CaCl_2 \cdot 1H_2O$	0.25 gm.	$CaCl_2$	0.155 gm.
Cane sugar	4.0 gm.	$FeSO_4$	0.0015 gm.
Bacto-peptone	0.5 gm.	Micro-element solution	1.0 ml.
Micro element solution	1.0 ml.	Distilled water to make	1.0 L.
Distilled water, to	1.0 L.	Adjust pH to 6.8 with KOH	

The particular micro-element solution used contained in 18 liters of distilled water: 30 ml. of 18 N HNO_3; $LiCl_2$, 0.5 gm.; $CuSO_4 \cdot 5H_2O$, 1.0 gm.; $ZnSO_4 \cdot 6H_2O$, 0.5 gm.; $Ti_2(SO_4)_3$, 1.8 gm.; $MnCl_2 \cdot 4H_2O$, 7.0 gm.; $NiCl_2 \cdot 6H_2O$, 1.0 gm.; $Co(NO_3)_2$, 1.0 gm.; KI, 0.5 gm.; KBr, 0.5 gm.; Na_2SO_4, 0.5 gm.; $K_2Cr_2O_7$, 0.2 gm.; $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 0.5 gm.,
Its use does not appear to be absolutely necessary when ordinary reagents are employed in making up the media with boron and manganese added (0.5 microgram/ml.).

The contents of a flask may be used directly as an algal cell suspension. When greater concentrations of cells are desired, the cells are concentrated by centrifuging. The usual procedure is to centrifuge out the cells in a graduated 15 ml. centrifuge tube, wash once or twice and resuspend them in whatever medium one wishes to use. For short term experiments no precautions against contamination are necessary. The dry weight, algal nitrogen or phosphorus can be obtained from an aliquot. Suspensions prepared in this manner can be used immediately, or, for certain types of experiments, they may be stored in an ice-chest for several days. The quantity required can readily be measured out with a pipette. Suspensions prepared in such a manner have been used in studying photosynthesis (Manning, et al. (1938); Petering, et al., (1939); Fan, et al., (1943)) and phosphorylation (Emerson, et al., (1944)). Most investigators working on photosynthesis and respiration employ algal cell suspensions prepared in somewhat the same manner (e. g., Emerson and Lewis, 1941; Gaffron, 1940; Pratt, 1943).

METHODS OF PREPARING MICROBIAL TISSUES

R. H. Burris

PREPARATION OF BACTERIAL CELLS

Washed bacterial cells have been widely used in studies of respiratory enzymes. Washing is considered to remove most nutrients and thus render the cells "non-proliferating" or "resting". Among the advantages rightly claimed for such preparations are: (a) various bacteria present a wide variety of enzymes for study; (b) the organisms can be readily grown under reproducible conditions; (c) the cells give a uniform suspension that can be pipetted accurately; (d) most washed cells can be stored for some time at refrigerator temperatures without appreciable change in activity; (e) respiration remains linear with time; (f) bacteria are extremely active per unit of tissue; and (g) gas diffusion into the cell is not a limiting factor normally.

The media required for best growth of bacteria vary widely with the organisms concerned. In the case of organisms which produce gums or capsules, it may be necessary to grow the cells on media which are low or lacking in carbohydrate to facilitate centrifugation and to limit the high endogenous respiration characteristic of cells abundant in reserve materials. Aside from such considerations, the cells should be grown on as favorable a medium as possible.

When small quantities of aerobic cells will suffice, they are most conveniently grown on an agar medium in liter Roux bottles. These bottles present a large area which is adequately covered by 75 to 85 ml. of agar. Inoculate the slanted, hardened agar surface with 2 or 3 ml. of a suspension of fresh cells, and distribute the suspension over the agar. After incubation, harvest the cells when they are young and active, even at the expense of a reduction in total crop. To harvest, add 10 ml. of buffer or mixed salt solution (e.g., Ringer's solution, Chapter 16) to each Roux bottle and scrape the organisms from the agar surface with a curved glass rod. Filter through cheesecloth to remove lumps of agar (by using 2% rather than the usual 1.5% agar less difficulty with breakage of the medium is experienced). Rinse with another 5 or 10 ml. portion of solution. Sediment the cells by centrifugation; resuspend in fresh solution and recentrifuge. Repeat. Place the washed cells in a tube or flask equipped for aeration, i.e., with a rubber stopper holding a glass tube leading to the bottom and an exit tube from the top Aeration at room temperature before the cells are used serves to exhaust metabolites and reduce the endogenous respiration. Store the cells at temperatures somewhat above freezing.

When anaerobes are to be used, they can usually be grown in stagnant carboys of liquid media and recovered by passage through a Sharples supercentrifuge, (Koepsell and Johnson, 1942). Precautions are frequently necessary to maintain reducing conditions during harvest to minimize inactivation of the enzymes.

Large quantities of aerobes quite generally can be produced in aerated liquid culture. Lee and Burris (1943) have described the growth of Azotobacter vinelandii under such conditions. Again the Sharples supercentrifuge provides a rapid means of handling large volumes of a culture medium.

The washed cell suspensions are adjusted by dilution to a point where they will induce a gas exchange of 100 to 200 µl. per hour in a Warburg flask; correspondingly, a dilution such that they will reduce methylene blue in 15 to 30 minutes on a suitable substrate is desirable for dehydrogenase studies. The first dilutions will be empirical, but activity can be correlated with turbidity measurements on a colorimeter, spectrophotometer, visual nephelometer, or a set of $BaSO_4$ tubes of varying turbidity. Subsequent suspensions can be adjusted to a reasonably constant activity by dilution to a standard turbidity. When measuring turbidity photometrically in the usual yellow bacteriological medium, it is customary to employ a red filter (620 or 660 mµ) to minimize the effect of the yellow interfering color. With washed suspensions of bacteria in the absence of such color it is advantageous to use a blue filter, since the instrument is more sensitive to turbidity changes under such conditions. Most stock bacterial suspensions will require about a 10 fold dilution to adjust the turbidity to a range suitable for standardization on a photoelectric colorimeter. For example, a suspension of Rhizobium trifolii is of

about the proper concentration when 1 ml. added to 9 ml. of water gives 45% transmittance in an Evelyn colorimeter at 420 mμ., distilled water being used as blank.

Turbidity serves as a means of judging activity for adjusting suspensions, but final cell activity is best expressed as the $Q_{O_2}(N)$, or μl. of oxygen taken up per hour per mg. nitrogen content of the cells. The relative merits of dry weight, total nitrogen, nucleic acid phosphorus, total carbon, and cell numbers as bases for expression of activity, are discussed by Burris and Wilson (1940) and in Chapter 1 of this book. In Chapter 10 directions are given for micro Kjeldahl determinations. Duplicate nitrogen determinations should give a basis for expressing $Q_{O_2}(N)$ values for all observations made with one particular suspension.

CELL-FREE ENZYME PREPARATIONS FROM BACTERIA

The advances in our knowledge of yeast fermentation, which have resulted from studies with cell-free preparations, emphasize the value of such experimental material. In their excellent review of methods that have been employed in producing cell-free bacterial juices, Werkman and Wood (1940) have enumerated the advantages and disadvantages of these preparations. Among the advantages are cited the facts that, the results are not affected by growth and reproduction of cells; cellular permeability is no factor in the measurements; it is possible to isolate and follow single reactions through the use of inhibitors and specific substrates; and it is possible to reconstruct a chain of enzymatic reactions by the combination of the individual components of the system. The disadvantages include the change in environment suffered by the enzymes detached from the cell, and the destruction or incomplete solution of certain enzymes.

Werkman and Wood (1940) should be consulted for a listing and description in some detail of all the methods which have been employed in liberating bacterial enzymes from the cell. In this discussion we shall confine our attention to methods in current, practical use that require only simple, readily obtainable equipment.

GRINDING WITH POWDERED GLASS

Wiggert, Silverman, Utter and Werkman (1940) describe a simple method for macerating bacterial cells by adding powdered glass and grinding with a mortar and pestle. The bacterial cells are best grown in liquid culture and recovered with a Sharples supercentrifuge, as in most cases several grams of wet cells are required. A subsequent wash and recentrifugation is often advisable to remove excess nutrients. The cells should be harvested while young and active; the loss in total crop by early harvest is largely compensated for in greater unit activity. Obviously the manner in which the bacteria are grown will vary with the organism concerned, and the individual investigator will be best acquainted with the nutrient and environmental requirements of the organism with which he is dealing.

Pyrex glass is powdered by grinding in a ball mill; 24 hours with stone balls but not over 4 hours with steel balls usually suffices for proper powdering. Sift the glass through a 30 mesh screen to remove large particles. When handling the glass powder wear a dust mask.

Mix 3 g. of bacterial paste, 25 g. of powdered glass and 7 ml. of pH 7.0, M/15 phosphate buffer; the consistency will be that of a firm batter. Place 10 g. of this mixture in a chilled 4.5 inch mortar and grind vigorously for 5 minutes. Do not use larger than 10 g. portions; observe the 5 minute grinding period, for additional grinding results in inactivation rather than increased yields. Combine the separately ground portions, and for each 10 g. portion add 2 ml. of phosphate buffer; mix well. Transfer to centrifuge tubes and sediment the glass by 5 to 10 minutes centrifugation at as great speeds as the equipment will allow. With particularly viscous preparations longer centrifugation may be necessary. When working with small quantities of viscous material, sedimentation directly in the Beams centrifuge followed by recentrifugation in the cleaned rotor may save time and give more complete recovery from the glass powder. Remove the supernatant and clarify by 2 to 15 minutes centrifugation in a Beams, Weed and Pickels (1933) "spinning top" air driven centrifuge equipped with a screw top with rubber gasket seal. Such a centrifuge can be constructed at low cost by any competent mechanician or instrument maker. The centrifugal force it will develop will depend largely upon the rotor diameter

and the pressure of air used for driving the rotor; hence, it is impossible to state exactly the time necessary for sedimentation. This can be determined empirically by observation of the degree of clarification with varying periods of centrifugation. With a 1.25 in. rotor Wiggert, et al. (1940) report suitable sedimentation in 2 minutes at 175,000 r.p.m.; this speed is obtained with 80-90 pounds air pressure. With a 1.5 in. rotor operating from a 30 pound per square in. air line, we obtain good clarification in 3 to 5 minutes with azotobacter preparations. Other preparations may require considerably longer sedimentation; cooling the juice is sometimes helpful in hastening sedimentation. Remove the supernatant with a pipette; the sediment is tightly packed on the walls of the rotor and is not appreciably dislodged when the rotor is decelerated evenly with the fingers.

In operating the Beams "spinning top" centrifuge the hollow rotor can be completely filled, since sedimentation appears to be as effective as when the rotor is only partially filled. Screw the top of the rotor "finger-tight" against the rubber gasket. Open the air line completely, so full pressure is applied to the stator and lower the rotor into position on the stator. Release the rotor, guiding it as it starts to rotate by having the fingers and thumb encircle it in an "eagle-grip". As the rotor gains speed and passes the "chatter-point", line it up by touching the edge gently with the thumbnail. To stop the rotor, leave the air pressure on at full force, place the fingers around the rotor in an "eagle-grip" and apply pressure gently from all sides. The air stream, upon which the rotor revolves, serves to cool the fingers, so there is no discomfort in stopping the rotor. Even deceleration of the rotor can be judged by the uniform drop in pitch of the audible note from the centrifuge.

Utter and Werkman (1942) described a modification of the manner of grinding given above. Glass and cells were prepared as usual, but were combined in the proportion of 2 parts of powdered glass to 1 part of bacterial cell paste. Buffer to give the consistency of a thick batter was added. "The grinding was accomplished by passing the bacteria-glass mixture between concentric ground glass cones. The inner cone was rotated by a motor". Such cones can be made from standard taper 24/40 or larger joints or built to specifications by any glassworker. If a specially built cone is employed it should have a taper of 30°-40°; by bringing the inner cone to a tip of about 1/4" diameter and constricting the junction between the outer cone and cell paste cylinder, cell loss can be minimized. To make the cones from stock standard taper joints, obtain the inner joint with a drip tip, and seal the tip off flat as close to the ground area as is possible without distorting the joint. Fill the inner joint and its length of tubing with crushed ice, and attach its open end to a motor (geared to about 300 r.p.m.) with a rubber stopper. Place the bacterial paste in the unground end of the standard taper outer joint. Join the outer and inner joints, which are mounted in a horizontal position, start the motor, and by means of a plunger, fashioned from a rubber stopper, slowly force the cell-glass paste between the rotating cones. Catch the extruded material in a chilled dish. Extract the paste with 1.5 ml. of water for each gram of bacteria used. Treat subsequent to this point in the same manner as described for preparations ground with a mortar and pestle.

Juices prepared in this way are essentially free from intact cells. They can be employed directly in respirometer vessels or Thunberg tubes with appropriate additions of buffers and substrates. Any considerable dilution should be avoided, as it may involve a greater than proportionate loss in activity. Wiggert, et al. (1940) discuss the activity of cell-free juices obtained from a variety of bacteria in relation to grinding time, volume of extracting fluid, buffers and substrates employed, addition of viable cells, storage and filtration. Juices from Aerobacter indologenes were completely inactivated by passage through Seitz, Jena glass, or Chamberland filters. With cell-free preparations of Azotobacter vinelandii, Lee, Burris and Wilson (1942) and Lee, Wilson and Wilson (1942) found very little inactivation of the enzymes examined after passage through a Berkefeld N or Mandler 15 filter.

The method of grinding with glass requires simple equipment, is successful with most organisms tested, and comparative studies indicate it has about the same effectiveness as the bacterial mill of Booth and Green (1938) in respect to both speed and completeness of cell disintegration. The Booth-Green mill will not be described here; it can normally be obtained from Unicam Instruments Ltd., Arbury Road, Cambridge, England.

AUTOLYSIS OF CELLS

One of the simplest means of preparing cell-free enzymes is by autolysis. The ease and the conditions under which different cells will autolyze vary widely. Some cells will autolyze while other cells are growing in the same culture medium, so that the filtered medium will at times carry a usuable concentration of freed intracellular enzymes. Many bacteria, however, are refractory to autolysis, or the autolysis requires such a prolonged time that many enzymes are inactived during the interval. Again the individual must determine the proper conditions for autolysis of the particular cells with which he is concerned.

Stephenson (1928) prepared cell-free lactic, succinic, and formic dehydrogenases from Escherichia coli. In studying the correlation of enzyme activity with number of viable cells present she found that aging suspensions increased in activity on lactate. This observation suggested that autolysis was freeing the enzyme, and optimum conditions for the autolysis were determined. The organisms were harvested, washed, suspended in pH 7.6, M/2 phosphate buffer and incubated at 37°C. for 5 or 6 days in a stoppered bottle. Addition of 1% sodium fluoride prevented putrefactive deterioration, but did not alter the enzyme. The preparation could not reduce molecular oxygen but could reduce methylene blue in the presence of any of the three substrates listed. Filtration through a porcelain filter resulted in complete inactivation. Passage through kieselguhr gave an almost water clear preparation which retained only lactic dehydrogenase activity.

To deal in general terms, it is customary to allow cells to autolyze in heavy suspensions under a layer of toluene at room or incubator temperatures. To determine optimum conditions, a series of samples of cells may be suspended in buffers at a variety of pH's. The supernatants should be tested at intervals to determine the time of greatest activity. After such a survey, a standard practice for autolyzing particular cells may be adopted.

LYSIS BY ADDED AGENTS

Lysis can be readily induced by the addition of foreign agents to certain sensitive species of bacteria. A notable example is Micrococcus lysodeikticus which is rapidly lysed by the addition of raw egg white, saliva, or tears to a suspension of the organism. Fleming and Allison (1924) found that a heavy suspension could be completely cleared in 30 seconds at 50°C. by the addition of 1% egg white. On incubation for 24 hours at 37° lysis was observed in a one to 50 million dilution of egg white.

Penrose and Quastel (1930) compared the enzyme activity of intact and lysed cells of M. lysodeikticus, and reported that lysis increased the rate of p-phenylenediamine oxidation, increased the activity of catalase, fumarase, and urease, left peroxidase activity unchanged, and destroyed or reduced the activity of the dehydrogenases for glucose, fructose, lactic acid, succinic acid, glutamic acid, and glycerophosphoric acid. Krampitz and Werkman (1941) grew M. lysodeikticus on a glucose, peptone, yeast extract medium, and washed and recovered the cells by centrifugation. To a 10% suspension of wet cells they added 1/10 volume of saliva, and incubated the mixture for 1 hour at 36°C. The cell-free supernatant exhibited an active oxalacetic acid decarboxylase.

In his studies of formic acid decomposition by Escherichia coli, Stickland (1929) found that autolysis would not liberate the enzymes involved. He resorted to digestion of the cells at 37°, pH 7.6, with crude trypsin, using 5 ml. of Benger's "liquor pancreaticus" to 100 ml. of cell suspension. Periodic tests for dehydrogenase activity showed an initial rise followed by destruction of lactic and succinic dehydrogenases, whereas formic dehydrogenase activity continued to increase. The treatment yielded a cell-free formic dehydrogenase, whose activity appeared to be associated with cell debris, but completely destroyed formic hydrogenlyase and hydrogenase thus preventing the production of H_2 and CO_2 anaerobically or H_2O and CO_2 aerobically from formate.

Though we can cite no case of its having been used for bacterial enzyme preparations, Sylvester (personal communication) has suggested the possibility of inducing lysis by bacteriophage. The lytic action is rapid and the treatment mild, hence one might anticipate that enzymes would be liberated with little inactivation.

FREEZING AND THAWING OF CELLS

Bacterial enzymes may often be released into solution by rupturing the cell with alternate freezing and thawing. By such a method Avery and Neill (1924) prepared a cell-free extract of pneumococci that formed peroxide when exposed to oxygen. The cells from a broth culture were recovered by centrifugation and suspended in one volume of phosphate buffer or nutrient broth for each 35 volumes of the original culture. This suspension of unwashed cells was placed in long, narrow tubes, sealed with vaseline, and alternately frozen and thawed rapidly 6 to 9 times. The cell debris was sedimented by 3 or 4 high speed centrifugations. The material did not lose its activity on passage through a Berkefeld filter under an atmosphere of nitrogen.

Koepsell and Johnson (1942), in their studies of the pyruvic acid metabolism of Clostridium butylicum, used a cell-free solution prepared by freezing the bacteria. Wet cell paste, as taken from the Sharples supercentrifuge, was packed in stoppered tubes and frozen immediately after harvest. While frozen, the cells slowly ruptured and released their contents. After 12 days 85 g. of cell paste was evenly suspended in boiled, cooled water, to give 250 ml. volume, and centrifuged. The supernatant liquid contained most of the original activity of the cells. This supernatant was dried under high vacuum and as a dry powder remained stable for some months.

DRYING CELLS

The permeability of cells frequently is sufficiently altered by drying, or the cells disrupted, so that enzymes may pass into solution when the dry powder is resuspended. This is the basis for the preparation of the many enzymatically active materials of "Lebedew" juice from yeast. Bernheim (1928) prepared a lactic acid dehydrogenase from baker's yeast by drying one pound of yeast in air for 8 to 10 hours, followed by drying with 4 successive 250 ml. portions of acetone, which were quickly removed by vacuum filtration. A 30 g. lot of the stable dry powder, when resuspended in 100 ml. of M/15 Na_2HPO_4 for 4 hours with intermittent grinding, released the lactic acid dehydrogenase into solution. Extraneous material was removed by centrifugation and dialysis.

Bovarnick (1941) demonstrated hydrogenase activity in an acetone preparation of E. coli. The washed cells were suspended in phosphate buffer equivalent to a thirtieth the original culture volume and poured into 20 volumes of cold acetone. The material was filtered quickly and dried under vacuum.

Apart from making cell-free preparations, the use of acetone for drying may alter the cell permeability to allow external metabolities to enter. Krampitz and Werkman (1941) demonstrated oxalacetic decarboxylase activity in acetone treated M. lysodeikticus, whereas the untreated cells did not decarboxylate the compound.

COMMENTS

It has been our purpose to emphasize means of preparing cell-free juices which require relatively simple equipment, to stress the methods we have personally employed, and to describe briefly and cite references to other procedures. Unquestionably the Booth-Green mill and properly designed supersonic apparatus will yield excellent enzyme preparations from bacteria, but the equipment necessary is not widely available. The powdered glass grinding method of Wiggert, Silverman, Utter and Werkman (1940) and the modification described by Utter and Werkman (1942) has much to recommend it, because of its general application and because it involves only mild treatment.

PREPARATION OF MOLD AND ACTINOMYCES TISSUES

Both molds and actinomyces normally grow as a heavy mat over the surface of media. This mat may be handled in much the same manner as animal tissues. It may be removed, washed with water and cut into slices. Semeniuk (1944) has used a process roughly equivalent to the homogenate technique in which the mycelium was ground with sand. The use of the homogenizer itself (see Chapter 9) has not been, in our experience, very satisfactory since most of the activity of the tissue was lost and various supplements had little effect.

A second technique which is used with both molds and actinomyces (Woodruff and Foster, 1943) is to grow the mycelium in a submerged state with aeration. This is done either by forcing sterile air through the medium or by continual shaking of the culture flasks. In the latter the usual procedure is to use about 100 ml. of medium in a 250 ml. flask (Erlenmeyer) and to place the inoculated flask in a shaker which operates with a 2 to 4 cm. stroke at a speed of between 60 to 100 cycles a minute. Under these circumstances "pellets" of mycelium are formed which can be pipetted readily. One precaution should be noted; such pellets may not have the same metabolism as the mycelium grown on the surface; for example, citric acid is not formed in appreciable quantity by Aspergillus niger under submerged conditions (see Tamiya, 1942). Various preparations of mold and actinomyces tissue may also be employed, especially in the study of phosphorylation. So far as present knowledge of phosphorylation extends it apparently differs from the known processes of animal tissues (Mann, 1943; Semeniuk, 1944), if, indeed, it exists in molds (Nord, Dammann and Hofstetter, 1936; Nord, 1939).

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Chapter Nine

THE HOMOGENATE TECHNIQUE

V. R. Potter

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Chapter Nine

THE HOMOGENATE TECHNIQUE

PRINCIPLES

The use of homogenates assumes for purposes of methodology that any chemical reaction which occurs in living cells will also occur in cell-free preparations of protoplasm; that this assumption is not unjustified has become evident during the eight years since publication of the original paper (Potter & Elvehjem, 1936). Homogenates consist of suspensions of the particulate components of protoplasm including mitochondria, microsomes (Claude, 1943), secretory granules, etc. plus a solution of the freely soluble diffusible components of protoplasm such as inorganic ions, coenzymes and carriers. If such preparations are properly made it is possible to dilute them until the endogenous oxygen uptake is virtually abolished, and then by the restoration of appropriate soluble factors at the proper concentration it is possible to study the reactions of any enzyme desired, to the exclusion of other enzymes whose cofactors or substrates have been eliminated by dilution. The method was devised in order to be able to assay quantitatively specific enzymes in the tissues of experimental animals. It was necessary to develop methods which would be applicable to whole tissue homogenates rather than to any extracts thereof, since any extraction method would be inexact because the completeness of extraction would be unknown. The homogenate methods are therefore designed to isolate a particular reaction, and, by so doing, to determine quantitatively the specific enzyme which catalyses the reaction. The methods have been applied to tissue samples as small as 13 mg. (immature rat ovaries) and are adapted to rapid manipulation. It remains to be seen how many enzymes can be studied by this technique, but several applications will be described.

In addition to the assay methods, the homogenate technique lends itself to the study of reaction mechanisms which involve a number of coordinated enzyme systems, and to the development of procedures for the testing and fractionation of new enzymes. By adding certain cofactors and substrates and omitting others it is possible to effect a reconstruction and integration of particular enzyme organizations which represent segments of cell function.

CONSTRUCTION OF THE HOMOGENIZER

The apparatus consists of a test-tube and a close-fitting power-driven pestle (see Fig. 22). The original homogenizer consisted of a 16 x 150 mm. pyrex test tube and a pestle which was made by sealing off one end of a 220 mm. length of 6 mm. capillary tubing and blowing a thick-walled cylindrical bulb about 20 mm. long, using a slightly larger test-tube for mold. Later it was found advantageous to form the bulb from a piece of thick-walled glass tubing whose outside diameter is that of the inside of the test-tubes. The large tubing is sealed to the capillary tubing, then constricted and sealed off at a length of about 20 mm., and molded in the test tube as before. For working with extremely small tissue samples, it is convenient to use small homogenizers, in which case 13 x 100 mm. pyrex tubes can be used, and the pestles can be blown from 6 mm. capillary tubing as in the original method. The final operation is the sealing of 6 or 7 small beads of about 2 mm. diameter to the bottom of the pestle to form cutting teeth. The device is then ready to grind. The beads are ground down on emery cloth so that each one has a flat surface which will approximate the inside of the bottom of the test tubes. This gives them a right-angled cutting edge which remains until the teeth disappear with continued use. Pestles with teeth less than 1 mm. high are best. It is desirable to have five or ten outfits on hand and to have a number of test tubes for each pestle. The sides of the pestles are ground with a few moments operation in an over-size test-tube containing a light suspension of fine carborundum powder in water. The tubes are ground similarly, using an undersize pestle. The object is not to produce a ground glass surface over the entire area of the grinding surfaces, but rather to eliminate all of the irregularities from both the tubes and the pestles. When the grinding is completed, pestles and tubes are matched by testing them with water in the tubes: the tubes should fall off the pestles

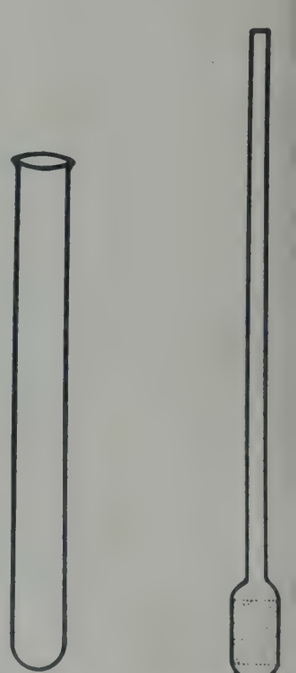

Fig. 22. Tissue Homogenizer.

very slowly when not supported. The clearance is then 0.10 - 0.12 mm. For any given pestle, it is well to have both tight-fitting and loose tubes, and as wear takes place, new tubes can be fitted to the pestle. (The presence of powdered glass in the homogenates can introduce serious errors into dry weight determinations where these are made by evaporating aliquots of dilute homogenates. M. O. Schultze has found that Lucite can be used to construct pestles, as it can be machined to any desired dimension. It was said that Lucite pestles do not erode glass from the test-tubes and that the resulting homogenates yield valid dry weight data).

In Cori's laboratory, the pestles have been made from stainless steel. We also use stainless steel pestles for certain operations such as grinding tissues in trichloracetic acid for analytical purposes, in which case loose fitting tubes are used. The bulb of the pestle was machined to size and threaded so that a 6 mm. threaded rod could be screwed into it. The bottom of the pestle was scored to form cutting teeth.

The homogenizer is powered by a cone-driven stirring motor of the Cenco or Sargent type, operated at a speed of about 1000 r.p.m. The use of a cone-drive motor prevents breakage because when a piece of connective tissue or muscle jams between the pestle and the tube, the friction drive slips instead of breaking the pestle.

THE USE OF THE HOMOGENIZER

Homogenates are usually not prepared until the reaction mixtures have been added to all of the vessels. The animal is usually killed by decapitation and the desired organ is quickly excised and placed in cracked ice (from distilled water) or in a Petri dish lined with moist filter paper. For assay purposes, a representative tissue sample should be taken. This is no problem in the case of most organs such as liver or muscle, but in the case of kidney and brain it is best solved by using one whole kidney or a specified lobe of the brain. Unless the amount of tissue is limited, it is well to take a sample weighing between 500 and 800 milligrams wet weight. The tissue can be accurately and speedily weighed on a torsion balance of one gram capacity graduated in fifths of a milligram, such as is made by the Roller-Smith Company.

The weighed tissue is dropped into a homogenizer tube which contains 1 ml. of distilled water and is homogenized in this volume until thoroughly blended; the remaining water is then added and the homogenization is completed. To make a ten per cent homogenate it is simplest merely to add 9 times the weight of the tissue (minus the one ml. which was originally in the tube). Very often more dilute homogenates are used. These are conveniently made by diluting an appropriate amount of 10 per cent homogenate, without further grinding. For some work it is unnecessary to chill the tissue, the homogenizer and the water. However this procedure is desirable in many cases, and we find it convenient to keep the motor as well as a 100 ml. graduate containing distilled water and a 5 ml. Mohr pipette in the cold room, while the homogenizer tube is kept in cracked ice when the tissue is being weighed and the calculations are being made.

The homogenizer functions by tearing up the tissue with the cutting teeth and then grinding it between the walls of the tube and the walls of the pestle. Accordingly, the tube is moved up and down rapidly in order to force the tissue back and forth past the grinding walls. The pestle is supported rigidly in a chuck on the drive shaft of the stirrer. Various investigators have used a short length of pressure tubing to effect a universal joint between the drive shaft and the pestle. This is not satisfactory for the production of cell-free homogenates unless the homogenizer tubes and pestles are almost perfectly matched. On the other hand, imperfectly fitting tubes and pestles can be used quite successfully when the pestle is supported rigidly, if the operator pushes the tube laterally while working it up and down so that the clearance between pestle and tube is zero on one side. One has to avoid breaking the pestle, of course.

The completed homogenate will be relatively cell-free but will contain shreds of connective tissue which tend to clog ordinary pipettes. It is advisable to use pipettes that have slightly enlarged openings at the end for pipetting homogenates. These are easily prepared when the tips are drawn out, an operation which should be done with all pipettes used for this type of work anyway. Mohr pipettes are used.

Homogenates should not be stored and used later on in the same day unless this fact is recorded with the data. Many enzymes which have been too labile to study thus far by other techniques can be studied by means of fresh homogenates.

PREPARATIONS

Thus far all the respiratory enzyme systems which have been studied by the homogenate technique have been found to require the addition of cytochrome c and it should be added whenever these systems are being investigated. Indeed, one of the main experimental developments which made it possible to develop the use of homogenates was the preparation of cytochrome c by Keilin and Hartree (1937) which is described in Chapter 16, page 188.

Just as cytochrome c can diffuse away from the particulate enzyme moieties, it is possible for other water soluble enzymes to migrate into the solution also. It is possible to prepare solutions of these enzymes which are free from the particulate components which function in the electron transmitting systems. These solutions contain the enzymes of glycolysis and are sometimes called Meyerhof extracts. They are also rich in the various coenzyme I dehydrogenases (Green, Needham and Dewan, 1937; Dewan and Green, 1938). Such preparations have been used to fortify homogenates in the study of triosephosphate dehydrogenase (Potter, 1940) and for the study of malic dehydrogenase and the coenzyme I-cytochrome c reductase (Potter, 1944). We have followed the method of Green, Needham and Dewan and found it satisfactory. Description of the preparation of these materials will be found in Chapter 16, page 192.

The study of interrelations between the respiratory enzymes and the phosphorylation systems can be conveniently studied by means of homogenates, provided the necessary components are present. One of the compounds that is most frequently needed is adenosinetriphosphate (ATP), because it is the common denominator for so many of the interlocking reactions of phosphorylation respiration. Furthermore, the compound forms the starting material for the preparation of its derivatives, which include adenosinediphosphate (ADP) and adenosine-5-monophosphate (adenylic acid), and the inosine series of compounds. Until recently the compound could not be purchased but it has now been placed on the market by Armour and Company at $35.00 per gram (latest quotation). (Experiments require about 2 mg. or less per flask). The methods for preparing the compound have been given by Lohmann and Schuster (1935) and by Needham (1942). Its preparation is described in Chapter 16, page 181.

Of the remaining substances that are needed for investigations involving homogenates, little can be said except to emphasize purity. We have obtained Merck products such as ascorbic acid, cysteine and other amino-acids whenever this has been possible. Merck also has been able to provide cocarboxylase. Pyruvic acid obtained from any source must be redistilled at 3-5 mm. Hg, preferably twice (Lipschitz, Potter and Elvehjem, 1938). The product should be diluted to approximately 1 Normal (addition of about 17 volumes of water; Sp. Gr. of pyruvic acid is 1.288). The diluted acid should be standardized, and adjusted immediately before using, by the addition of the theoretical amount of bicarbonate solution to a diluted pyruvic acid to give a final concentration of 0.1 N, and the pH should never rise above 6.8 during the neutralization. Robertson (1942) has purified pyruvic acid by precipitating the sodium salt from alcohol, see page 105. Potter and Schneider (1942) purified sodium succinate ($\cdot 6H_2O$) by alkaline precipitation of metals and recrystallization. Malic acid can be obtained from Pfanstiehl or Eastman. Citric acid is readily available but iso-citric acid is not. However, Pucher (1942) has obtained the latter in appreciable yield from blackberry leaves. Hexosediphosphate can be obtained from Schwartz Laboratories, Inc., see page 178 and glutathione from the B. L. Lemke Company.

RESPIRATORY ENZYMES

Succinic dehydrogenase and cytochrome oxidase. The technique for assaying animal tissues for these two enzymes was described by Schneider and Potter (1943). The assay for cytochrome oxidase was carried out on the same tissue samples as the assay for succinic dehydrogenase, and it was found that in all cases the oxidase was present in wide excess over the dehydrogenase. This means that when succinate is the substrate the limiting factor is the enzyme (or enzymes) which oxidize succinate and reduce cytochrome c, that is, succinic dehydrogenase. On the other hand, when cytochrome c is reduced chemically,

the limiting factor is the enzyme (or enzymes) which brings about the reaction between cytochrome c and oxygen, that is, cytochrome oxidase. Although both enzymes may consist of more than one component they may be treated as single entities for assay purposes. The sequence of reactants in the two cases shows the relation of the two assays.

Succinic Dehydrogenase assay: (Rat Liver $Q_{O_2} = 88$)

Succinate → Succinic Dehydrogenase → Cytochrome c → Cytochrome Oxidase → O_2

Cytochrome Oxidase assay: (Rat Liver $Q_{O_2} = 392$)

Ascorbate → Cytochrome c → Cytochrome Oxidase → O_2

In every tissue thus far examined the preponderance of cytochrome oxidase over succinic dehydrogenase has been great enough to make the succinic dehydrogenase assay a valid one. One would expect the cytochrome oxidase system to have a greater capacity for electron transfer than any one of the systems which funnel into it, and the fact that such is the case makes it technically possible to measure the capacity of the component systems. The assay has been arranged so that one sample of tissue can be assayed for both enzymes using six flasks and manometers set up as follows, using rat liver as an example:

TABLE XVI

Reaction Mixtures and Results in the Succinic Dehydrogenase-Cytochrome Oxidase Assay (37°C.)

The center cups contained 0.1 ml. 2N NaOH + 1 Sq. cm. folded filter paper; Warburg flasks without side arms; 10 minutes equilibration.

Flask No.	1	2	3	4	5	6
	ml.	ml.	ml.	ml.	ml.	ml.
H_2O (to make 3.0 ml.)	0.6	0.5	0.9	0.3	0.25	0.20
0.1 M PO_4 pH 7.4 with NaOH	1.0	1.0	1.0	1.0	1.0	1.0
0.5 M Na-Succinate pH 7.4	0.3	0.3	0.3	--	--	--
1×10^{-4} M. Cytochrome c	0.4	0.4	--	--	--	--
2.4×10^{-4} Cytochrome c	--	--	--	1.0	1.0	1.0
4×10^{-3} M. $CaCl_2$	0.3	0.3	0.3	--	--	--
4×10^{-3} M. $AlCl_3$	0.3	0.3	0.3	0.3	0.3	0.3
0.114 M. Na-ascorbate pH 7.0	--	--	--	0.3	0.3	0.3
1% rat liver homogenate	--	--	--	0.10	0.15	0.20
5% rat liver homogenate	0.1	0.2	0.2	--	--	--
μl O_2 uptake per 10 min. (average of four 10 minute periods)	20.0	42.0	8.0	30.4	43.2	56.0

The plan does not include a measurement of the endogenous oxygen uptake of the liver because experience has shown that this is insignificant. However, it is well to establish this point in any new work, especially if larger amounts of tissue are used. The amount of tissue is usually chosen so as to give oxygen uptake values of approximately the range indicated. The treatment of the data will now be described:

Succinic dehydrogenase: The necessary data are given by flasks 1 and 2. The assay is always run at two levels of tissue rather than with duplicates at the same level, because this technique has the advantage of providing continual proof that the uptake is proportional to the tissue concentration. The data are reported finally as Q_{O_2}, that is, the microliters of oxygen taken up per hour per milligram of dry weight. However, the

data are first converted to the 10/20 ratio, which is the average oxygen uptake per 20 mg. of fresh tissue per 10 minutes (or 10 mg. fresh tissue per 20 minutes). Since the readings are taken at 10-minute intervals and the homogenates are prepared in strengths of 1, 5, or 10% the 10/20 ratio can usually be calculated mentally, and its usefulness lies in the fact that it is identical with the Q_{O_2} when the per cent dry weight of the tissue is 30%. This is the value most frequently found for rat liver. To illustrate, the data from flasks 1 and 2 are used to obtain the 10/20 ratio and to calculate the Q_{O_2}:

$$\text{The 10/20 ratio} = (4 \times 20) + (2 \times 42) / 2 = 82$$

$$\text{The } Q_{O_2} = 82 \times 6 \times 1/20 \times 100/30 = 82$$

When the per cent dry weight is not 30, the 10/20 ratio is multiplied by $\frac{30}{\text{per cent dry wt.}}$ to give Q_{O_2}. The Q_{O_2} should be defined according to the substrate which is used. The data from flasks 1 and 2 therefore give the succinate Q_{O_2}.

Cytochrome Oxidase: It has been found that whole cells in homogenates seem to be impermeable to the substrates which are used in the cytochrome oxidase assay. The best solution to this difficulty is to have the degree of homogenization as complete as possible. As an alternative, one can calculate the per cent homogenization on the assumption that the uptake on succinate when no cytochrome is added is due to whole cells. This may not be completely valid but it gives at least an approximation. The per cent homogenization is obtained from flask 3 and the 10/20 ratio from flasks 1 and 2. The 10/20 ratio for flask 3 is 16, and for flasks 1 and 2 it was 82. The per cent homogenization is then calculated to be $100 - \frac{16 \times 100}{82} = 100 - 19.5 = 80.5\%$.

The cytochrome oxidase assay is complicated by the fact that the substrates are all more or less autoxidizable and are generally affected by traces of copper or catalysts other than the cytochrome system. We selected ascorbic acid as the best reductant for the system, but it also has some autoxidation, the measurement of which cannot be made simply by putting the substrate in a flask with the buffer and measuring the rate of oxidation. We have found that the best way to measure the autoxidation rate is to extrapolate to zero tissue concentration from a series of three different tissue concentrations as in the table, in which flasks 4, 5 and 6 contain 1.0, 1.5 and 2.0 mg. of fresh liver, respectively. The autoxidation rate is obtained in this case by subtracting the increments in the last two flasks from the value of the first flask:

$$30.4 - (12.8 + 12.8) = 4.8$$

This value is then subtracted from all the values, and the 10/20 ratios are calculated. The oxygen uptakes corrected for autoxidation are then, respectively, 25.6, 38.4 and 51.2 and the 10/20 ratios are 256, 256 and 256 and the uncorrected ascorbate Q_{O_2} is therefore 256, which is corrected for whole cells to give a corrected ascorbate Q_{O_2} of 318.

The conditions which have been described as optimum for this enzyme system probably apply to other tissues as well, but should not be assumed to do so.

Malic dehydrogenase and CoI-Cytochrome C-reductase: It appears likely that these two enzyme systems can be assayed by utilizing the cytochrome system as the terminal connection with oxygen, since the cytochrome oxidase seems to be present in considerable excess (see preceding section). Handler and Klein (1942) showed that coenzyme I is rapidly broken down in homogenates and one might expect that the study of coenzyme systems might be impossible in homogenates. However they also showed that the breakdown is inhibited by nicotinamide, as had been reported by Mann and Quastel (1941). Oxalacetate, the product of malate oxidation, powerfully inhibits the reaction and unless it is removed the system cannot be studied. Its oxidative removal, besides being difficult to accomplish, would complicate the results. The oxalacetic acid is therefore removed by means of the transaminase reaction, which Straub (1941) utilized for this purpose. Transaminase is present in the homogenate. The reaction sequence is

Malate—malic dehydrogenase—Coenzyme I—CoI-cytochrome c-reductase—cytochrome c—cytochrome oxidase—oxygen.

The underlined reagents are added in excess; the side reactions are controlled by adding nicotinamide and glutamate; the limiting factor is malic dehydrogenase when the cytochrome reductase is present in excess. In order to assay for the reductase, one must add malic dehydrogenase in excess; this can be done by adding a Meyerhof extract, the preparation of which has been described (page 94). The system appears to require a higher concentration of cytochrome c than does the succinate system. An assay can be carried out for both enzymes in duplicate using 4 flasks and manometers as follows, with no controls described. The removal of oxalacetate is still not 100% efficient and this probably accounts for the rapid slowing of the reaction. The data is therefore based on the first two five-minute readings, while a third reading is taken to show that the reaction has not slowed appreciably in the first fifteen minutes. The system has so far been studied only in rat liver, and although the reactants are believed to be present in optimum concentration, the system described is given only as an example.

TABLE XVII

Reaction Mixtures in the Malic Dehydrogenase and Coenzyme I-Cytochrome c reductase assay (37°C.)

(0.1 ml. 2N NaOH + 1 sq. cm. filter paper in center cup)

Flask No.	1	2	3	4
	ml.	ml.	ml.	ml.
H_2O	0.7	0.6	0.1	--
0.1 M. PO_4 pH 7.4 with NaOH	0.8	0.8	0.8	0.8
0.1 M. Nicotinamide	0.3	0.3	0.3	0.3
0.5 M. Na-malate	0.3	0.3	0.3	0.3
0.5 M. Na-glutamate	0.3	0.3	0.3	0.3
0.5% CoI (side arm; added after equilibration)	0.2	0.2	0.2	0.2
4×10^{-4} M. cytochrome c	0.3	0.3	0.3	0.3
Meyerhof extract, excess	--	--	0.6	0.6
5% rat liver homogenate	0.1	0.2	0.1	0.2

The malate Q_{O_2} is calculated from flasks 1 and 2 and the CoI Q_{O_2} is calculated from flasks 3 and 4; the 10/20 ratio is based on the first ten minutes of oxygen uptake.

Keto-acid oxidases: The oxidation of ketoglutaric acid and pyruvic acid has been successfully carried out in homogenates but the optimum conditions cannot yet be described. The following factors all need to be tested and their proper concentration determined before an assay can be described: adenylic acid, magnesium ions, cocarboxylase, oxalacetate, glutathione, coenzyme I, ATP, and sodium chloride. The system may require coordination with the phosphorylative system for maximum rates, although this is not a requirement of the succinate system.

THE COUPLING OF OXIDATION AND PHOSPHORYLATION

Adenosinetriphosphatase: The logical preliminary to the study of the coordinated phosphorylative and oxidative systems was a knowledge of the rate of dephosphorylation of the phosphorylated compound which has thus far been the immediate product of the phosphate donating reactions, that is, adenosinetriphosphate. DuBois and Potter (1943) devised an assay and applied it to normal rat tissues. It was found that the calcium ion was a necessary co-factor in all tissues studied, and the assay consists in measuring the rate of inorganic phosphate released when the enzyme is saturated with calcium ions and ATP, with conditions such that the rate of phosphate release is proportional to time and to tissue concentration. The assay is carried out in a small volume to conserve ATP, using 10 x 50 mm. test tubes. However, the reaction may be carried out in a larger volume by keeping all the reactants at the same final concentration per unit volume. The assay is always carried out at two levels of tissue rather than in duplicate, since this provides a control on the technique.

TABLE XVIII

Reaction Mixture for ATP-ase assay, (37°C)

Tube No.	1	2	3	4	5
	ml.	ml.	ml.	ml.	ml.
H_2O	0.20	0.10	0.45	0.25	0.30
0.5 M. diethylbarbiturate pH 7.4	0.15	0.15	0.15	0.15	0.15
0.04 M. $CaCl_2$	0.05	0.05	0.05	0.05	0.05
.013 M. ATP, pH 7.4	0.15	0.15	--	--	0.15
1% rat liver homogenate (added 2 or 3 minutes after the tubes are placed in the thermostat)	0.10	0.20	--	0.20	--

The reaction is stopped by adding 0.1 ml. of 50% trichloracetic acid to each tube. The tubes are then centrifuged 10 minutes at 3000 r.p.m. and 0.3 ml. of the supernatants is carefully drawn off for analysis for inorganic phosphate. The reagent blank on the phosphate analysis is used for the I_0 readings, (100 setting on Evelyn colorimeter) and should include 0.3 ml. of the supernatant from tube no. 3. Tube no. 4 gives the inorganic phosphate from the tissue and no. 5 the spontaneous breakdown of the ATP. The assay is based on the amount of inorganic phosphate liberated from ATP per milligram of tissue in 15 minutes and is obtained from tubes 1 and 2 after applying the corrections obtained from tubes 3 and 4. The phosphate is determined by the Fiske-Subbarow method, as described on page 163 of this book.

The oxidative synthesis of phosphate bonds: It is possible to carry out oxidations over the cytochrome system and to use this energy for the esterification of inorganic phosphate using adenylic acid or ADP to accept the phosphate from the oxidized intermediates. However, ATP-ase assays as described in the preceding section show that any ATP formed will be split and inorganic phosphate will be returned to the medium, and the inorganic phosphate of the medium will not decrease unless the esterification can outpace the ATP-ase. If creatine or some other phosphate acceptor is added it is possible to tap off the ATP phosphorus and to minimize the amount of ATP available for dephosphorylation. Creatine seems to be ideal for this purpose. In addition, the action of ATP-ase is retarded by fluoride and magnesium ions, which can be added to the mixture, since fluoride does not inhibit the oxidations appreciably at the concentration used, and magnesium appears to be necessary for the phosphorylation. The complete optimum system has not been worked out but it has been adequately established that inorganic phosphate can be esterified using cell-free tissue homogenates at remarkably low concentrations. This data is as yet unpublished but will be included here, so as to be conveniently available. It must be emphasized that tissues are excised quickly and cooled on ice; they are homogenized rapidly and in the cold, and they are added to reaction mixtures held at zero degrees with all reactants present. As soon as the homogenate is added, the flasks are attached to manometers and placed in the 37° bath. They are equilibrated for 10 minutes, oxygen uptake is measured for 10 minutes and the flasks are then taken from the manometers and placed in cracked ice; 2 ml. of 17.5% trichloracetic acid is used to stop the reaction. The flask contents are transferred to centrifuge tubes and centrifuged in the cold and kept in cracked ice when not in the centrifuge. The phosphorus distribution can be studied according to the methods of chap. 15; when a volume of 3.0 ml. is used in the final tube containing reduced phosphomolybdate, we use 0.05 ml. and 0.10 ml. of the protein-free supernatant fluid for the analysis for inorganic P + phosphocreatine P. To determine "true" inorganic P, 2.0 ml. of protein-free supernatant fluid are brought to pH 8.3 (phenolphthalein) with 2N NaOH and 0.4 ml. of the calcium reagent is added. The precipitate is centrifuged down, drained by inversion and the excess supernatant fluid blotted with filter paper. The precipitate is suspended in water to give 1.8 ml. and dissolved by adding 0.2 ml. 2N HCl. The solution is then equivalent to the original, and "true" inorganic phosphate is determined in aliquots of 0.05 ml. and 0.10 ml. The phosphate content of the original flask is obtained by multiplying by 100 and by 100/2 respectively. The original P content is determined by adding the enzyme after the 2 ml. trichloracetic in one case. In these experiments ATP is added originally. As it reacts with phosphocreatine, ADP and adenylic acid are made available to the oxidative P-donors.

It seems undesirable to have any more adenylic acid exposed to its deaminase than can be avoided. When the oxidative systems do not phosphorylate, the inorganic phosphate increases due to ATP breakdown, but when the system contains all the components, a net phosphate uptake is observed. This reaction is especially strong in kidney cortex homogenates but it also occurs in other tissues. A system which gives vigorous P uptake with only 20 mg. of cell-free homogenized normal rat kidney cortex per flask is as follows:

TABLE XIX

Contents of System Capable of Oxidative Phosphorylation

Solid Creatine	30 mg.
H_2O	1.5 ml.
0.5 M KCl	0.2 ml.
0.1 M $MgCl_2$	0.2 ml.
0.25 M NaF	0.2 ml.
0.10 M PO_4 pH 7.4 with NaOH	0.1 ml. (=310 micrograms P)
2.0×10^{-4} M Cytochrome c	0.2 ml.
0.0133 M ATP	0.2 ml.
0.5 M Na-Succinate pH 7.4	0.2 ml.
10% rat kidney cortex homogenate	0.2 ml.

The system will also esterify inorganic phosphate when malate and oxalacetate are used instead of succinate, though not at the same concentration; between 0.1 and 0.2 ml. of 0.05 M. malate or oxalacetate should be used.

The preparation of systems which can maintain their energy reservoir (ATP) will probably make it possible to study reactions hitherto considered "vital" and not open to study by cell-free techniques.

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Chapter Ten

CHEMICAL METHODS FOR USE WITH WARBURG FLASKS

Manometric methods find their widest use in the study of tissue metabolism. At times that metabolism consists of many reactions whose substrates or products do not involve gaseous change. At other times reactions measurable by gas exchange have associated with them other reactions not so measurable. It is thus decidedly convenient to have available chemical methods to determine the disappearance of substrates, the appearance of products, or the change in one or more of the substances in the medium in which the tissue is suspended. We are here concerned with the available chemical methods for analyzing the contents of respirometer flasks. The use of the respirometer as an instrument of chemical analysis is described in Chapters 11 and 14.

Chemical methods suitable for estimation of the contents of respirometer flasks are relatively few in number. Certain limitations are responsible for excluding a large number of otherwise satisfactory procedures. Since the manometric methods described are capable of measuring very small changes, chemical methods employed must be capable of measuring changes of the order of 5 to 10 micrograms. Their total range must usually be from 0 to 100 micrograms. The small quantities involved exclude all but the simplest fractionation procedures, so that any methods employed must be specific. In addition they must usually be specific enough to measure small quantities of the compound involved without interference by other related materials which may be present in larger quantities.

For practical purposes colorimetric methods are the only ones available. Colorimetric methods, including turbidimetric and nephelometric methods, for a wide variety of compounds have been described by Snell and Snell (1937), and clinical handbooks, manuals of methods, etc. abound in descriptions. An examination of the recent literature will often disclose methods suitable for use with the respirometer.

It is not our purpose to describe these. We are concerned only with basic methods which would probably be used daily by one working with manometric techniques. It is assumed that the use of the colorimeter is adequately known. The methods are described for the Evelyn (1936) photoelectric colorimeter, but of course are easily adapted to other instruments. In the case of some micro-modifications a smaller type tube is convenient. These are described in Chapter 15.

PREPARATION OF TISSUE FOR ANALYSIS

W. W. Umbreit

The usual procedure for the analysis of the contents of respirometer flasks involves stopping the reactions at the desired point. Usually this is done by adding, at the appropriate time, sufficient trichloracetic acid to yield 5-10% final concentrations. 100% solutions of trichloracetic acid are easily prepared (100 grams trichloracetic acid made to 100 ml.). Thus in a flask containing 3 ml. of fluid, 0.3 ml. of 100% trichloracetic acid may be added (tipped in from side arm, etc.) at the end of the measured interval. This stops the reactions, precipitates the proteins, and frequently extracts materials from the cells. The contents of the flask are removed, centrifuged, the clear supernatant fluid used for analysis.

USE OF THE COLORIMETER

The method should first be run on pure compounds. Standard curves relating color to quantity of the compound should be prepared. In general, in using the Evelyn photoelectric colorimeter (1936) one selects a portion of the curve which shows a straight line relationship between the quantity of the compound and the "2-log galvanometer reading" value. The number 2 (= log of 100) represents the galvanometer reading in the complete absence of the color being measured. Hence, 2 - log G, or log 100 minus the log of the galvanometer reading represents the amount of light absorbed by any given degree of color. If the logarithm of the amount of light absorbed bears a straight line relationship to the amount of the material to be estimated, the absorption follows Beer's law. In determining unknown samples a standard is included from which the contents of the samples are calculated. This standard must agree with the standard curve within the limits of precision of the method. The precision quoted is the standard deviation from the mean of a series of duplicates. All methods described below follow Beer's law over the range indicated and are not subject to great fluctuation.

METHODS FOR NITROGEN

Ammonia: (Modified from the method of Johnson (1941)). Make sample to 10 ml. Add 5 ml. 2N NaOH (or 2N KOH) and 2 ml. Nessler's Reagent. Mix, read after 10 min. (or longer) in the colorimeter using a 490 mμ. filter. Range: 10-100 micrograms nitrogen; precision ± 1.5 micrograms. Micro-modification: Make sample to 2 ml. Add 2 ml. 2N NaOH and 1 ml. Nessler's Reagent. Read in colorimeter using a 420 mμ. filter (use standard curve because of slight departure from linearity). Range: 2 to 20 micrograms of nitrogen. Precision: ± 0.8 micrograms.

Reagents: Nessler's Reagent: Grind 5 grams of KI and 2-3 grams HgI_2 in a mortar with 50 ml. of water and make to 500 ml. Allow this to stand for several days; filter to remove excess HgI_2. To 500 ml. of the filtrate add 160 ml. of a 1.5% gum ghatti solution, mix, and dilute to 1500 ml. Solution stable for years. It is convenient to prepare this material in large batches (10 to 20 liters) and to dispense it with a burette in a permanently assembled apparatus.

Total Nitrogen: Pyrex glass test tubes are employed. Digest the sample in a sand bath (150-170°C) with 1 ml. digestion reagent for 12 hours or longer. This is to evaporate the water from the sample and prevent bumping when heated later. Heat over a microburner with 1-2 drops 30% H_2O_2 until fuming has ceased. Cool. Wash into colorimeter tube, dilute to 10 ml. and proceed as in method for ammonia (or develop color in digestion tube and transfer to colorimeter tube later). When relatively large quantities of organic matter are present a longer digestion is necessary. Precision: ± 1 microgram. Rapid modification: If the sample contains from 10-100 micrograms of nitrogen and occupies a volume of less than 1 ml., the following method may be employed. Add sample to pyrex test tube. Add 0.2 ml. conc. H_2SO_4 and 2 drops 30% H_2O_2. Heat over micro-burner until fumes cease, cool, wash into colorimeter tube, dilute to 10 ml. and proceed as in method for ammonia. Precision: ± 2.0 micrograms. Micromodification: Add sample (1.0 ml. or less) to pyrex test tube. Add 0.2 ml. digestion reagent. Digest over microburner until rapid fuming ceases (3-10 minutes). Cool. Dilute to 2 ml. Add 2 ml. 2N NaOH. Add 1 ml. Nessler's reagent. Pour into dry colorimeter tube and read using 420 mμ filter. Range: 2 to 20 micrograms nitrogen. Precision: ± 0.5 micrograms. Reagents: Digestion reagent is 5 N H_2SO_4 with 150 mg. copper selenite per liter. Method also used in Chapter 15.

METHOD FOR GLUCOSE AND OTHER SUGARS

Glucose is determined by the method of Folin and Malmros (1929) in which the sample is made to 4 ml. in a colorimeter tube, 2 ml. of 0.40% $K_3Fe(CN)_6$ and 1 ml. of carbonate-cyanide mixture added. The sample is mixed, heated 8 minutes in a boiling water bath, cooled 1-2 minutes and 5 ml. ferric iron solution added to produce the color. The volume is made to 25 ml. with distilled water, mixed, and the color read using a 520 mμ filter. Range: 10-100 micrograms glucose equivalents. Precision: ± 0.4 micrograms.

Reagents: Carbonate-cyanide: Dissolve 8 grams anhydrous Na_2CO_3 in 40-50 ml. water, add 15 ml. freshly prepared 1% NaCN. Dilute to 500 ml. Stable for long periods. Ferric iron: Soak 20 grams gum ghatti in one liter of water for 24 hours. The gum is suspended in the water in a cheesecloth bag. Add a mixture of 5 grams anhydrous $Fe_2(SO_4)_3$, 75 ml. 85% H_3PO_4 and 100 ml. water. After mixing add slowly about 15 ml. of 1% $KMnO_4$ to destroy reducing materials present in the gum ghatti and allow the solution to stand for a few days. Stable for long periods.

The range of this method can be narrowed and its precision somewhat increased by measuring the color at 470 mμ. This method is actually one measuring "reducing value" rather than glucose as such and is reported under the term "reducing value" in Chapter 15. Details of detection of other sugars and their estimation are given by Bates (1942) and by Gurin and Hood (1939, 1941). Determinations for pentose (ribose, arabinose, xylose) and for fructose are given in Chapter 15.

METHODS FOR PHOSPHORUS

This may be determined by methods described in Chapter 15. If a slightly longer range is desired the following procedure may be employed:

The sample is mixed in a colorimeter tube with 2 ml. 2.5% ammonium molybdate made up in 5 N H_2SO_4 (or 1 ml. each of 5% molybdate and 10 N H_2SO_4), diluted to about 20 ml., 1 ml. reducing reagent added, and diluted to 25 ml. Range: 10-100 micrograms phosphate-phosphorus. Precision: ± 0.3 micrograms. Micromodifications are easily devised by a proportionate reduction of all reagents.

METHOD FOR LACTIC ACID

G. A. LePage

This compound can be conveniently and accurately determined by the method of Barker and Summerson (1941). Certain practical details and convenient modifications which have been found desirable in our experience are described below:

Procedure: Measure a sample, (containing from 5 to 50 micrograms of lactic acid) approximately neutral in reaction, into a clean test tube and make to a volume of 4.5 ml. Add 0.5 ml. of 20% $CuSO_4 \cdot 5H_2O$. Add approximately 0.5 gm. of $Ca(OH)_2$, and disperse the precipitate by shaking. One can use clean stoppers and shake the tubes vigorously, but with the small volume in an ordinary test tube, one can dispense with use of stoppers and mix well by tapping the tube, in an almost horizontal position, on the palm of the hand. The copper-calcium hydroxide precipitate formed removes all interfering materials. The precipitate should be redispersed several times in the course of 30 minutes or more, then centrifuged. Transfer a 1.0 ml. sample of the supernatant to a clean colorimeter tube. Care must be taken not to include any of the precipitate from the surface film that remains after centrifugation. Chill sample and tube in an ice bath. From a fine tipped burette, slowly add 6.0 ml. of concentrated H_2SO_4. The first third of the acid must be added slowly, with vigorous shaking, to avoid local heat development which causes irregular results (resulting in further oxidation of the acetaldehyde to acetic acid). Barker and Summerson (1941) report that cooling of the tube is unnecessary, but we have found that such cooling is essential to obtain consistently accurate results. After H_2SO_4 has been added, heat the tubes in a boiling water bath for 5 minutes (3-10 minutes is the possible range without change in results). Cool the tubes to below 30°C and add one drop, 0.05 ml. (by using the same dropper each time, including the determinations for the preparation of the standard, one need not measure these reagents except by drops), of 4% $CuSO_4 \cdot 5H_2O$ and one drop of p-hydroxydiphenyl reagent. Immediately disperse the reagent by shaking. Incubate at 28-30°C. for 30 minutes or more, with occasional shaking to disperse the reagent. Heat the tubes 90 seconds (60-120 is possible without change) in a boiling water-bath and cool. Read the color in Evelyn or other photoelectric colorimeter with a 560 or 565 mμ filter.

A standard curve can be run using pure zinc lactate or using C. P. 85% lactic acid. In using the latter, one should dilute to approximately 1 N and boil for 10 minutes to depolymerize. Then titrate with standard NaOH and phenolphthalein to determine the concentration and dilute appropriately. The amount actually determined in the color reaction is 1/10 that in the original sample, i.e., 0.5-5 micrograms. Results can be made accurate to ± 1 to 2%.

Reagents: Concentrated H_2SO_4: This should be C. P. grade and kept free of metals, organic matter and nitrates. Occasional batches of acid containing traces of nitric cannot be used. Burettes should be lubricated only with the acid; no grease.

p-hydroxydiphenyl. A 1.5% solution is made by dissolving the compound in 5% NaOH and diluting to 0.5% NaOH. Eastman grade of this chemical is suitable without further purification, but can be recrystallized from alcohol if desired. The solution of the reagent in 0.5% NaOH keeps for long periods at room temperature if in a colored stoppered bottle.

Remarks: A blank should always be run through the complete procedure to ensure that no contamination has occurred from glassware or reagents.

Necessary precautions in the use of this method involve care that the apparatus is clean. Contact of the fingers with the glassware where it will be in contact with the sample (lip of tube, etc.) must be avoided, since there is sufficient lactic acid on the fingers to markedly change results. Glassware cleaned in chromic acid must be washed

under the tap and immersed in a dilute alkali solution (strong soap or 1/5N NaOH) for a few minutes before final washing, to rid it of chromium. Otherwise traces of chromium remaining on the glass interfere. As soon as the operator becomes convinced of the necessity for the precautions outlined (especially cleanliness of glassware and slow addition of acid to the sample in the cold) consistently accurate results are easily obtained.

METHOD FOR PYRUVIC ACID

H. A. Lardy

Pyruvic acid is readily determined in biological materials by the method of Lu (1939) as modified by Bueding and Wortis (1940) and Elgart and Nelson (1941). When pyruvic acid is to be determined in blood it is essential that it be stabilized immediately by the addition of iodoacetate to a final concentration of 0.2%. The iodoacetate prevents the loss of blood pyruvate but care should be taken to deproteinize the blood as soon as possible in order to prevent an increase in pyruvate. Suspensions of cells or tissue homogenates which have been used in manometric experiments may be pipetted directly from the respirometer flask into trichloracetic acid.

Reagents: Solutions are given as grams of solute per 100 ml. final volume of solution. (1) 25% solution of iodoacetic acid in water adjusted to pH 7.8 with sodium hydroxide. (2) 10% trichloracetic acid. (3) 0.1% 2-4-di-nitro-phenylhydrazine in 2 N HCl. (4) Ethyl acetate. (5) 10% sodium carbonate. (6) 2 N NaOH.

Procedure: Three ml. whole blood (drawn into a tube containing sufficient iodoacetate to give a final concentration of 0.2%) are added slowly with continual shaking to 12 ml. 10% trichloracetic acid in an Erlenmeyer flask. Tissue suspensions from Warburg flasks are added to 4 volumes of trichloracetic acid. After standing for a few minutes the precipitate is filtered or centrifuged off.

Three ml. of the clear filtrate (or supernatant fluid) are added to 1 ml. of the 2-4-di-nitro-phenylhydrazine solution is a conical centrifuge tube. After standing at room temperature for 10 minutes, 4 ml. of ethyl acetate are added and the two layers mixed (preferably by bubbling a stream of nitrogen through a capillary pipette whose tip rests lightly on the bottom of the tube). After mixing, the layers are allowed to separate and the lower one (water) is carefully drawn off (with the same pipette) and transferred to a second centrifuge tube. The aqueous layer (in the second centrifuge tube) is extracted twice with 2 ml. portions of ethyl acetate and the extracts added to the 4 ml. of ethyl acetate in the original tube. The aqueous layer should now be colorless and may be discarded. The combined ethyl acetate extracts are treated with exactly 2 ml. of 10% sodium carbonate. The layers are mixed (preferably with nitrogen) for several minutes. After the layers have separated the sodium carbonate layer is quantitatively transferred to another tube and the extraction of the ethyl acetate repeated twice using exactly 2 ml. of sodium carbonate each time. The combined sodium carbonate extracts are then extracted once with 1 ml. of ethyl acetate, the latter removed, and the carbonate extract transferred to a colorimeter tube. Four ml. of 2 N NaOH are added and the contents mixed. The color is read in 10 minutes in a photoelectric colorimeter with a filter having the maximum transmission at 520 mμ. Range: 5 to 35 micrograms. Precision: ± 2 micrograms.

A calibration curve is obtained by the use of freshly distilled pyruvic acid (see page 94 and page 179) as a standard. Where large numbers of samples are to be analyzed, a series of tubes fitted into an aeration train and nitrogen bubbled through them all simultaneously is convenient.

If acetoacetic acid is present in the material to be analyzed it may be eliminated by adding 1/10 of its volume of concentrated HCl to the protein-free filtrate and heating in a boiling water bath for 1 hour (Elgart and Nelson, 1941). Concentrated NaOH (equivalent to the HCl) is then added, the solution cooled, and analyzed as described.

Pyruvic acid may also be estimated colorimetrically by the salicylaldehyde method of Straub (1936) or manometrically using carboxylase (see Chapter 14).

METHOD FOR CITRIC ACID

H. A. Lardy

This compound has always been most difficult to estimate. It may, however, be determined colorimetically with a rather high degree of accuracy. In principle, the citric acid is oxidized with potassium permanganate in the presence of bromine, under controlled conditions and is thus converted into pentabromoacetone; this may be measured by the color produced upon addition to sodium sulfide. Pucher, et al. (1934, 1936, 1941) and Purinton and Schuck (1943) have proposed specific quantitative methods based on this principle. All of these require the quantitative extraction of the pentabromoacetone and employ other rather involved procedures. The following method is somewhat simpler and only a single extraction is made. A single extraction was found to remove a constant amount of the total pentabromoacetone in any series of samples of uniform volume. The method is described by Perlman, Lardy and Johnson (1944).

Reagents: (1) Sulfuric acid: equal volumes of 95% sulfuric acid and water. (2) 1 M KBr. (3) Saturated bromine water. (4) 3% H_2O_2. (5) Petroleum ether (acid washed "Skelly Solve B"). (6) Dioxane-water mixture (equal volumes of dioxane and water). (7) Sodium sulfide solution (4 gms. $Na_2S \cdot 9H_2O$ per 100 ml. solution). (8) 1.5 N potassium permanganate. (9) "Weak" permanganate (0.1 N).

Preparation of samples: If the samples are known to contain reducing materials, aliquots (preferably containing less than 25 mg. citric acid) are placed in 1" x 8" pyrex test tubes, 2 ml. sulfuric acid added and the total volume adjusted to about 20 ml. After boiling for a few minutes, the solutions are cooled and 3-5 ml. of bromine water added. After 10 minutes any precipitate which may form is removed by centrifugation. The supernatant liquid is decanted off and made to 25 ml. If the samples do not contain appreciable amounts of reducing materials this treatment may be omitted.

Procedure: Aliquots of the sample (containing between 0.2 to 2.0 mg. citric acid in a volume of 3.5 ml. or less), are placed in test tubes (the 18 by 150 mm. size is convenient) graduated at 5 and at 10 ml. 0.3 ml. sulfuric acid, 0.2 ml. KBr and 1 ml. of the 1.5 N permanganate are added, the total volume being adjusted to about 5 ml. The tubes are allowed to stand for 5 minutes at room temperature and then chilled in an ice bath. The excess permanganate is decolorized with hydrogen peroxide (care must be taken to keep the reaction mixture below 5°C. during this step). Any excess peroxide is removed with "weak" permanganate. The total volume is now adjusted to exactly 10 ml. and 13 ml. of petroleum ether is added. The tubes are stoppered, shaken vigorously and centrifuged (to break any emulsion that may form). Ten ml. portions of the petroleum ether extract are added to colorimeter tubes containing 5 ml. water-dioxane and 5 ml. sodium sulfide solution. The colorimeter tubes are stoppered, shaken vigorously and centrifuged. The color produced should be a light yellow and will be fully developed in 5 minutes. It is stable for several hours. The absorption (in the aqueous (bottom) layer) is measured at 400-450 mμ., usually the 420 filter is used. A tube containing no citric acid, but which has gone through exactly the same procedure is used as a blank. The content of citric acid is calculated from at least two standards (at different levels) which are run with each set of samples. Range: 200-1800 micrograms citric acid. Precision: ± 10 micrograms.

Precautions: If too large a sample of citric acid has been used, a somewhat red (rather than yellow) color will be developed. In this case a smaller aliquot of the petroleum ether may be added to the dioxane-water-sulfide thus avoiding another complete analysis. When only small quantities of the material are available for analysis, the preliminary acid and bromine treatment may be carried out in a volume of less than 5 ml. in which case the whole sample may be treated with permanganate. The following are critical points in the procedure: (1) An excess of H_2O_2 or permanganate must not be present in the solution before the petroleum ether extraction. Excess H_2O_2 gives low recoveries, excess permanganate gives high recoveries. (2) The solutions must be thoroughly chilled before the excess permanganate is removed or erratic results will occur. (3) Some stabilizing agent must be present during the formation of the colored reaction product of the pentabromoacetone and the sodium sulfide. Both 50% dioxane-water and 50% pyridine-water have proven satisfactory. (4) Sometimes the petroleum ether contains interfering materials. These can be removed by washing with acid. (5) The pentabromacetone should not be allowed to remain in the petroleum ether for more than 15 minutes.

Isocitric acid, cis-aconitic acid, trans-aconitic acid, oxalacetic acid, and gluconic acid do not interfere with this method.

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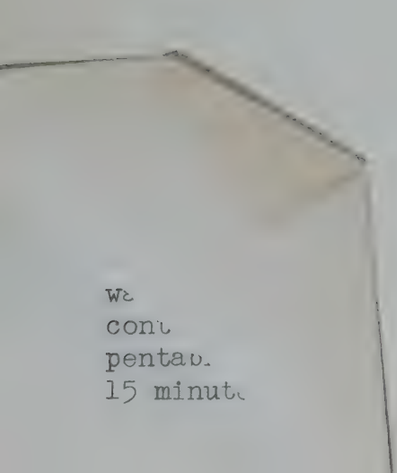
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Chapter Eleven

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Chapter Eleven

SPECIAL METHODS EMPLOYING MANOMETRIC TECHNIQUES

In most of the methods listed below some sort of specialized apparatus other than that normally accompanying a respirometer is required. In general, the methods have been devised to meet specific needs not covered by the usual instruments or to enable a measurement which is difficult and time-consuming on the normal instrument, to be done with more convenience. The method is described in principle only and references to detailed descriptions are cited.

THE "FIRST" METHOD OF DICKENS AND SIMER

W. W. Umbreit

In this method oxygen uptake is first determined by absorbing the CO_2 in alkali. At the end of the experimental period acid is added to the tissue and to the alkali thus liberating all of the CO_2 which can then be measured. A second manometer is used in which all of the CO_2 is so liberated at the start of the experimental period. Hence by this method one can determine:

(1) The oxygen uptake (and its rate) over the experimental period.
(2) The CO_2 liberated over the entire experimental period.

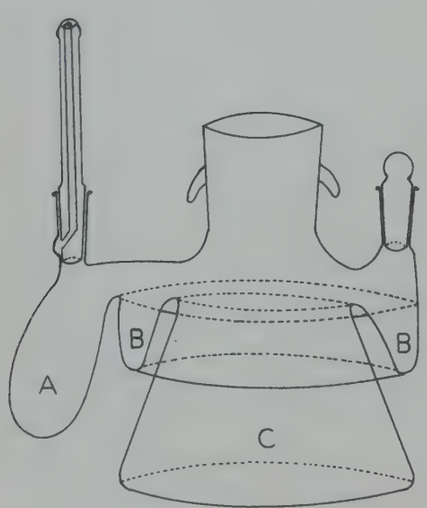


Fig. 23. Flasks for use with the "First" Method of Dickens and Simer. A-Sidearm containing acid. B-Trough encircling flask containing barium hydroxide. C-Main compartment.

Flasks of the type shown in Fig. 23 are recommended. Acid is placed in sidearm (A) (0.3 - 0.5 ml. 3N HCl or H_2SO_4). Alkali is placed in the trough (B) (usually 0.5 ml. cold saturated $Ba(OH)_2$ or M/5 to M/10 solutions of other alkalis, relatively free from carbonate). Upon shaking, the alkali swirls about in the trough with little tendency for it to splash over. The respiring tissue is placed in the main part of the flask (C). The method is quite useful but has the following limitations:

(1) Bicarbonate solutions cannot be used because the measurements are made in the absence of CO_2 in the air.
(2) The tissue is respiring in the absence of CO_2, which may affect the rate or course of metabolism.

Detailed descriptions are given by Dixon (1943), Dickens and Simer (1930, 1933) and Meyerhof and Schmitt (1929).

THE "SECOND" METHOD OF DICKENS AND SIMER

W. W. Umbreit

The basic principle of this method is that after an appropriate interval acid is added to the tissue liberating all bound CO_2 in the form of gaseous CO_2. The CO_2 in the gas phase is then all absorbed by the addition of alkali. Appropriate controls are used which permit one to measure oxygen uptake, acid production, and CO_2 production in buffers, bicarbonate, serum, etc. or mixtures of these. The actual description of the method is quite complex but it is certainly desirable to have some knowledge of the principles involved since, when equipment is available permitting its use, the method eliminates many of the complexities and uncertainties inherent in other methods, although it does introduce a few of its own.

The essential measurement is the CO_2 liberated by acid and the CO_2 absorbed by alkali. Two types of apparatus are available for this measurement. First the type of flask used by Dickens and Simer will be described. This is a two compartment flask illustrated in Fig. 24, fitted with sidearms, one of which tips into one compartment (i.e., A' into A); the other tips into the second compartment (B' into B). The tissue is placed in compartment A and acid in A'. Upon tipping A' into A the CO_2 fixed in the solution is released.

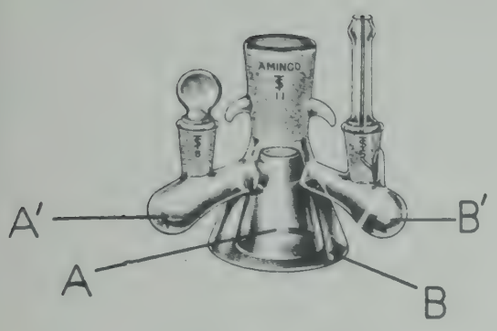


Fig. 24. Flask for use with the "Second" method of Dickens and Simer.

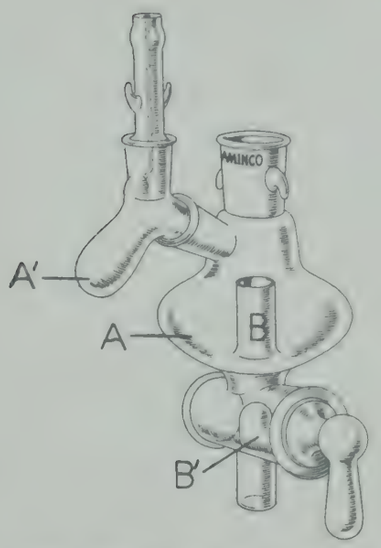


Fig. 25. Dixon-Keilin Flask (see text for description).

Compartment B contains M/5 $KMnO_4$ in M/500 H_2SO_4 while the sidearm (B') contains 30% NaI (acidified to M/500 H_2SO_4 just before use). Upon tipping B' into B mixing of the iodide with the permanganate results in an alkaline reaction which absorbs the whole of the CO_2 from the gas phase.

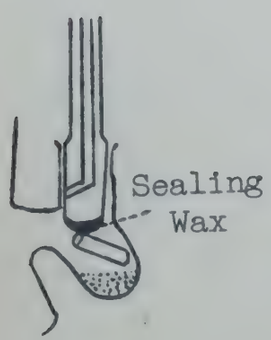
A second type of flask which accomplishes the same purpose is that used by Dixon and Keilin, illustrated in Fig. 25. Tissue is placed in compartment A, acid in the sidearm A'. Alkali is placed in the stopcock insert B' which when turned to a point parallel with the center well (B) permits the alkali to enter the flask. "B" usually contains filter paper to increase the surface of the alkali and a glass rod which, by dropping into the stopcock insert, displaces the alkali into B so that it wets the paper.

Measurement of respiration, CO_2 output and acid production are made with one flask but two others are required as controls. Details of the procedure have been adequately described in several places (Dixon, 1943; Dickens and Simer, 1933); the method is briefly as follows: In the center compartment of the principal flask, the tissue is suspended in phosphate, in other buffers, or in serum and is allowed to respire for a definite measured interval. At the end of this interval acid is tipped in from the side arm, and the gas so released (CO_2) measured (= final "bound" CO_2). After the readings are constant, the iodide is tipped into the permanganate (or alkali added in the Dixon-Keilin flasks) causing the absorption of CO_2. This is measured (= final CO_2 in gas phase). Oxygen uptake is determined by the drop in the reading after all the CO_2 has been absorbed compared to the initial reading. This drop was caused by (a) oxygen uptake, and (b) the absorption of the CO_2 which was in the gas phase at the start. A control flask is used to determine how much CO_2 was in the gas phase at the start, hence the oxygen uptake can readily be calculated. The CO_2 in the gas phase not accounted for by the initial CO_2 in the gas, plus that derived from the bicarbonate (a second control flask measures initial "bound" CO_2) is that produced directly by respiration or fermentation. The decrease in bicarbonate is a measure of the acid production, but it must be corrected for "acid retention" (see later) in order to give the true acid produced. The relatively large changes in gas pressure which occur (i.e., when bicarbonate is acidified, or all the CO_2 is absorbed) usually necessitate the use of mercury or Clerici's solution in the manometer. While the method is quite useful it has two limitations:

(1) "Acid retention" must be estimated and corrected for.
(2) It only serves to measure the reactions over an interval and does not provide for a continuous measurement of respiration, CO_2 output and glycolysis, as has sometimes been supposed.

Undoubtedly this method deserves more attention than has been accorded to it. In our opinion the necessity of specialized apparatus has been a serious limitation in its use. A modification which permits one to use the method with ordinary Warburg flasks having two sidearms is the following:

Tissue, buffers, etc., are placed in the main compartment. Acid is placed in one sidearm. The other sidearm usually contains 0.3 ml. saturated $KMnO_4$ in M/1000 H_2SO_4. Suspended above it is a tube of KI (solid) attached to the stopper with sealing wax (Fig. 26a, see Vogler, 1942). When, after the acid has been added, it is desired to absorb the CO_2 from the gas phase, the stopper is turned to break off the tube containing the KI; the KI mixes with the permanganate and causes an alkaline reaction whereupon the CO_2 is absorbed from the gas phase. The absorption is slow, however, and 8-12 hours are required for completion. This does not seriously affect the use of the method since the tissue is not metabolizing. The manometers are usually left in the bath (shaking slowly) overnight. Mixing of KI and $KMnO_4$ also can be accomplished by the use of a flask having one of its sidearms equipped with a double sack (Fig. 26b).



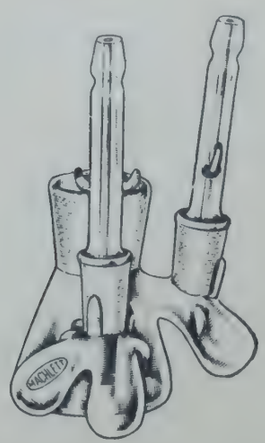


Fig. 26a Fig. 26b
Arrangements permitting the mixing of two materials in a sidearm (see text for description).

DIXON-KEILIN METHOD

W. W. Umbreit

This method is adequately described by Dixon (1943) together with several modifications. It is essentially the second method of Dickens and Simer but has been considerably simplified for use with the differential manometer. When this apparatus is available it is undoubtedly a very excellent method. Two flasks (Dixon-Keilin type) of exactly the same size (their size may be equalized by adding glass rods (Summerson, 1939)) and containing identical amounts of tissue, buffers, gas, etc. are attached to opposite sides of a differential manometer. At the start, acid is tipped into one of the flasks, giving the initial "bound" CO_2. Respiration, glycolysis, etc. continue in the other flask, causing a drop in the manometer. At the end of a measured interval acid is tipped into the second flask causing the liberation of "bound" CO_2 still remaining. Both flasks have now had the same treatment except that one has respired for a measured interval. Alkali is now introduced into both flasks simultaneously to absorb the CO_2 in the gas phase. From the resulting change in the manometer, the oxygen uptake can readily be determined. From the other readings mentioned one may determine the total CO_2 output and the change in "bound" CO_2. From the latter, by correcting for "acid retention", the acid produced may readily be calculated. Dixon (1937), Brekke and Dixon (1937), Elliott and Schroeder (1934) have described methods based on the Dixon-Keilin method. Dickens and Greville (1933) and Summerson (1939) have provided essentially similar methods but with improvements possible by the use of specialized apparatus. Dixon (1943) has discussed these in some detail.

RETENTION

W. W. Umbreit

If one studies cell metabolism in the presence of relatively large quantities of protein or other materials which can react with CO_2, the measurements made are inaccurate because some of the CO_2 has combined chemically with the protein, etc., and has not taken part in the gas exchange. Any acid formed has reacted partly with the protein and partly with the bicarbonate present. This CO_2 or acid which does not appear in the gas phase is termed "retained" CO_2 or acid and the phenomenon is spoken of as "retention".

When free CO_2 is liberated in a solution containing protein (serum, etc.) a part of it is bound first of all by the protein and then in lesser quantity by phosphates and other materials. The general equation of such binding ("CO_2 retention") is:

$$(45)\quad CO_2 \rightleftharpoons H_2CO_3 + S^- \rightleftharpoons HCO_3^- + SH$$

Where S^- represents the anions of the serum, other proteins, or other buffers present.

An acid (HA) reacts under the same circumstances partly with the bicarbonate present (liberating gaseous CO_2), partly with anions from the medium as follows:

$$(46)\quad HA + KCO_3^- \rightleftharpoons KA + HCO_3^- \rightleftharpoons CO_2$$

$$(47)\quad HA + S^- \rightleftharpoons A^- + SH$$

The reaction expressed by equation (47) is termed "acid retention".

Before going further it should be emphasized that both the production of free CO_2 and acid are equivalent to the production of H^+ ions, since $CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$. We must deal with a material, in equilibrium with CO_2 in the gas phase, in which acid (H^+ ions) are being produced but in which this H^+ production does not result in an equivalent CO_2 liberation because some of the H^+ has united with protein or other buffers and not with bicarbonate. In reality the problem is one of measuring the CO_2 liberated as gas and then determining what per cent of the CO_2 which was produced was converted into gas. From this we can calculate the true CO_2 production, in short:

true CO_2 and acid produced	=	CO_2 measured as gas by manometer (from CO_2 as such or from acid reacting with bicarbonate)	+ acid ions (from CO_2 or acid) which did not produce CO_2 in gas phase.

The theory for calculating retention and its measurement is very complex (see, for example, Warburg (1926), Krebs (1933), etc.), but with the experience gained over the years a relatively simple conception of retention and relatively simple methods for its measurement are now available. These have been described by Dixon (1937, 1943), Warburg, Kubowitz, and Christian (1931), Dickens and Simer (1933), Brekke and Dixon (1937) and others.

The essence of these methods may be described as follows (they each differ in details of technique and frequently in the method by which they reach the following conclusion):

Since only a certain percentage of the CO_2 liberated escapes into the gas phase, measurement of this gas is thus too low. However, if one could find what per cent of the acid produced did appear as CO_2 and what per cent did not, one could calculate the true acid production (carbonic + other acid) from these percentages; i.e., if 90% of the acid or CO_2 produced by the tissue appeared as gaseous CO_2, and if 10 µl of CO_2 were measured manometrically, the true CO_2 production would be $10/0.9 = 11.1$ µl.

One can calculate the amount of CO_2 liberated in respiratory or fermentative processes by multiplying the change in height on the manometer (h) by the flask constant (k_{CO_2}). When there is appreciable CO_2 retention, the h observed is less than should be the case. This could easily be corrected for by altering the flask constant to take care of this retention, i.e.,

$$(48)\quad X_{CO_2} = h(k_{CO_2} + V_F\, r) = hk^s_{CO_2}$$

Where "r" represents the retention, which, of course is dependent upon V_F, the amount of fluid in the flask (r = µl CO_2 retained per ml. medium). This is sometimes condensed into $k^s_{CO_2}$ which means the constant to be applied when retention occurs (the "s" stands for "serum").

The problem now becomes one of determining the value of "r". We have so far spoken of retention as being due to the union of carbonic acid and other acids with the buffering (usually protein) anions. And we have treated these as though the two types of acid were equivalent. But unfortunately, for purposes of calculation, they are not the same. If carbonic acid is produced and hydrogen ions from it unite with buffer cations, bicarbonate ions are left in solution, i.e., the bicarbonate concentration <u>increases</u>. If other acids

are produced, the hydrogen ions liberated from these combine with buffer cations and with bicarbonate, thus decreasing the bicarbonate concentration.

We must then separate r (retention) into the two types involved.

let: r_C = retention due to carbonic acid production

r_A = retention accompanying the production of other acids

Equation (48) thus becomes:

$$(49)\quad X_{CO_2} = h(k_{CO_2} + V_F r_C + V_F r_A) = hk^s_{CO_2}$$

It is unlikely that both types of retention will be encountered simultaneously as expressed in equation 49. The r values in the equation can be measured separately as follows:

Determination of Retention Due to Carbonic Acid Formation (r_C): A flask of the type shown in Fig. 24 (the flask shown in Fig. 26b will also serve) is required. Two of these are convenient. These are set up as follows:

Flask 1	Flask 2
Bicarbonate solution Omit medium 0.1 ml. M/20 lactic or citric acid	Bicarbonate solution 1 to 2 ml. medium to be studied 0.1 ml. same acid
gas mixture same in both	
(The bicarbonate concentration governs the gas mixtures employed, see Chapter 3.)	
After equilibration tip in acid from sidearm B' into $NaHCO_3$ solution in chamber B (Fig. 24); no acid is added to chamber A which is empty in flask 1 and contains medium in flask 2. The addition of acid liberates an identical amount of CO_2 from the bicarbonate in each flask; some of this is taken up by the medium in flask 2.	
The amount of CO_2 liberated from the bicarbonate is $X^1_{CO_2} = h^1k^1_{CO_2}$ (the "1" indicates the flask. Note that this flask need not be of the specialized type.)	In this flask some of the CO_2 has been absorbed by the medium. The observed CO_2 is $X^2_{CO_2} = h^2k^2_{CO_2}$

In flask 2, $X^1_{CO_2}$ µl of CO_2 was liberated but only $X^2_{CO_2}$ µl of CO_2 was observed. The amount of retained CO_2 is dependent upon the pressure increase in the second flask (h_2) and upon the volume of the medium in flask 2. The r_C (retention due to CO_2 production, per mm. Brodies increase in CO_2 pressure, per ml. medium) =

$$(50)\quad r_C = \frac{X^1_{CO_2} - X^2_{CO_2}}{h_2 V_F} = \frac{h^1k^1_{CO_2} - h^2k^2_{CO_2}}{h_2 V_F}$$

This value may be substituted in equation (49).

Determination of Retention Accompanying Other Acid Formation (r_A): This may be determined by adding a known quantity of acid to the medium, determining the amount of CO_2 liberated, and comparing this with the amount of CO_2 liberated from the addition of the same amount of acid to bicarbonate. Ordinary Warburg flasks equipped with sidearms are used.

Flask 3	Flask 4
chamber - - bicarbonate solution sidearm - - citric or lactic acid; must be less than equivalent to $NaHCO_3$	bicarbonate solution + medium to be studied acid; same as flask 3
gas mixture (+ CO_2) is the same in both flasks	
after equilibration, tip in acid in both	
CO_2 liberated from bicarbonate = $X^3_{CO_2} = h^3k^3_{CO_2}$	observed CO_2 liberated = $X^4_{CO_2} = h^4k^4_{CO_2}$

The amount of CO_2 retained is thus:

$$X^3_{CO_2} - X^4_{CO_2} = h^3k^3_{CO_2} - h^4k^4_{CO_2}$$

r_A= retention in µl per mm. (Brodie's) of CO_2 (produced by acid) per ml. serum =

$$(51)\quad r_A = \frac{h^3k^3_{CO_2} - h^4k^4_{CO_2}}{h^4v^4_F}$$

However, while the addition of the same quantity of acid would liberate the same amount of CO_2, the pressure of CO_2 would be different in flasks of different gas volumes. Also the change in bicarbonate concentration would vary with the fluid volume. Hence r_A ("acid retention") is dependent not only upon the buffering capacity of the medium (as is r_C) but also upon the volume of the flask and upon the volume of fluid employed. If one wishes to apply this estimate of "acid retention" to flasks other than the one in which it was determined, rather complex calculations are necessary. Hence, it is convenient to determine the "acid retention" directly on the actual flasks to be used (however, see below). But more important it is usually possible to determine the acid retention while the respiration or fermentation is in progress. This is done by adding a known quantity of acid, determining the amount of CO_2 it liberates and from the amount it would have liberated if added to bicarbonate, the retention can be calculated. Dr. Bain describes below the system for determining acid retention which he has used in measuring ribonuclease activity by means of acid production (Bain and Rusch, 1944).

MEASUREMENT OF ACID RETENTION

J. A. Bain

Warburg flasks, so designed as to allow the addition of two substances to the system at different times are required. Flasks with two sidearms were found to be convenient. In one sidearm is placed a measured amount of standard citric acid (0.1-0.2 ml. M/10). The flask is then placed in an oven at 75°C until the acid is completely dried. This is done in order to avoid changes in volume when the standard acid is added to the system. In the other sidearm is placed the requisite amount of substrate solution while the main part of the flask contains the tissue to be studied, the $NaHCO_3$ buffer, and water to make a total volume of 3.0 ml. The flask is gassed and equilibrated in the usual manner and the substrate tipped in. The rate of CO_2 evolution is measured for two five minute periods; at the end of the second period the dried acid is washed into the main part of the flask and three more readings are taken. The first two and the last two readings give the steady rate of the system. The difference between this rate and the third reading give the amount of carbon dioxide evolved by the standard acid. The variation between this value and the value obtained by tipping standard acid into $NaHCO_3$ alone allows a calculation of the retention correction to be made. The amount of acid introduced into the system is not large enough to change the pH appreciably and thus the activity of the tissue is not affected. The correction obtained is a function not only of the buffering capacity of the medium, but also of the volume of the flask and the amount of the gas evolved.

In the conventional method (Dixon, 1943) each flask is calibrated individually. A method has been devised whereby any number of flasks may be calibrated for retention from the data obtained by the use of just one flask provided the volume of each is known.

If the flask constants (k) are calculated for a series of flasks in the usual manner and plotted against the gas volumes (V_g) a straight line, hereafter referred to as the base line, is obtained. Suppose that a medium which retains carbon dioxide is introduced into these flasks. Each flask will now have a constant which is equal to k plus an amount "r" which will vary with each flask. If these new k values be plotted against V_g as before, a straight line will again be obtained, but will be found to lie above the base line and to have a different slope. The characteristics of this line are reflections of the facts that the amount of retention is a function of the buffering capacity of the medium, the volume of the flask, and the amount of gas evolved.

By introducing a given medium into three flasks of different volumes and measuring the amount of CO_2 given off when a known amount of standard acid was added, a retention line was determined directly. This was done with several media of different retentions and the data plotted (see Bain and Rusch, 1944). It was found that the slope (m) of these lines was proportional to the value of k at a given volume, i.e.:

When V_g is constant:

$$(52a) \qquad k_I m_{II} = k_{II} m_I$$

$$(52b) \qquad k_I m_{III} = k_{III} m_I$$

$$(52c) \qquad k_I m_{IV} = k_{IV} m_I$$

With this fact established it became apparent that the retention line for a given medium could be determined from the data of one flask if the base line and the volume of the test flask were known. The method of arriving at this conclusion is demonstrated as follows:

From Chapter 1, $k = x/h$, where k = the constant of the test flask of volume V_g, x = the theoretical µl. of carbon dioxide evolved by n ml. of standard acid, and h = the manometer reading produced by n ml. of standard acid.

Solving the established proportion for m, we find $m = km_{base}/k_{base}$, where m is the slope of the retention line, m_{base} the slope of the base-line and k and k_{base} the constants of the flask of volume V_g at retention and at base-line levels.

By solving a simple analytical equation for a straight line, we find $k' = m(V'g - Vg) + k$, where k' is the constant of the flask of volume V'g at retention line level.

With V_g known and V'g arbitrarily assigned, k and k' can be calculated from the above equations and the retention line (thus defined by the points (k, Vg) and (k', V'g)) constructed. The constant for any apparatus volume may now be read directly from the retention curve for the medium involved.

The use of these retention values in the Warburg "indirect method" (Chapter 4) is described by Dixon (1943), in the second method of Dickens and Simer (Chapter 11) by Dixon (1943) and Dickens and Simer (1932), and in the Dixon and Keilin method (Chapter 11) by Dixon (1937, 1943).

MEASUREMENT OF GASES OTHER THAN OXYGEN AND CARBON DIOXIDE

R. H. Burris

Hydrogen: Hydrogen exchange may often be involved in bacterial reactions. In the oxidation of hydrogen with molecular oxygen (the Knallgas reaction) a combination of oxygen and hydrogen takes place and difficulty arises in establishing how much of each gas reacts. If the reaction can occur at low partial pressure of oxygen the individual gases taken up in the overall reaction can be measured as described by Lee, Wilson, and Wilson (1942). Employing a mixture of 98% H_2 and 2% O_2 in the Warburg flasks they allowed the reaction to proceed to a point where the oxygen was exhausted. The oxygen supplied was accurately measured in independent flasks containing pyrogallol and alkali, the alkali being added from the sidearm after temperature equilibrium was reached. Such a method is limited to gas mixtures containing not over 2.5% of oxygen. Subtracting the pressure change in the oxygen analysis flask (alkaline pyrogallol) from the total pressure change in the flask containing H_2 and O_2 indicated the pressure change attributable to H_2 uptake.

If hydrogen evolution is to be measured in an anerobic system for which independent analysis has demonstrated that hydrogen and carbon dioxide are the only gaseous products, the carbon dioxide may be absorbed with KOH in the inset cup and the gas pressure attributed to hydrogen production. Woods and Clifton (1937) have followed such hydrogen output with simultaneous estimation of carbon dioxide evolution in independent flasks.

Nitrogen: In the biological reactions of nitrogen fixation and denitrification molecular nitrogen is involved. The amounts of N_2 reacting are usually relatively small, but can be measured by manometric methods.

Nitrogen fixation may be followed directly in Warburg respirometers in the following way: The Brodie's fluid in the manometers is replaced with mercury which has been moistened with water; 1 to 5 mm. of water is kpet above each mercury column to minimize the sticking of the mercury in the capillary manometers. The biological agent to be studied is introduced into a special Warburg flask arranged so the contents of the inset alkali well can be poured into the sidearm of the vessel, or provided with a double sidearm permitting mixing of two components in the sidearm (Fig. 27). The cultures are shaken in the Warburg bath in the ordinary manner, and pure oxygen is added to the flasks when needed as indicated by the manometer. If tank oxygen is used the amount supplied must be recorded so a final correction may be made for N_2 added with the O_2. After it is judged that the biological agent has accomplished its nitrogen fixation, the alkali from the center well of the vessel is poured into the sidearm which contains pyrogallol (slightly acidified to limit spontaneous oxygen absorption) or the contents of the double sidearm flask mixed, and the manometer is shaken until equilibrium is reached. The absorption of oxygen will yield the percentage of oxygen initially present in the flask if no nitrogen has been fixed, however, if nitrogen has been fixed its removal will appear as an apparent increase in the oxygen content of the flask. For example, if the gas mixture initially supplied were air with 21% oxygen, and the final absorption of oxygen indicated that 23% of the gas supplied had disappeared, one would conclude that 2% of nitrogen had been fixed. Such measurements may prove useful in establishing nitrogen fixation by materials which by virtue of a high initial nitrogen content cannot be accurately analyzed with the Kjeldahl procedure. The method has been applied by Allison, Hoover and Minor (1942) and in our laboratories; the theory of the method as used with Novy-Soule respirometers is discussed by Hurwitz and Wilson (1940).

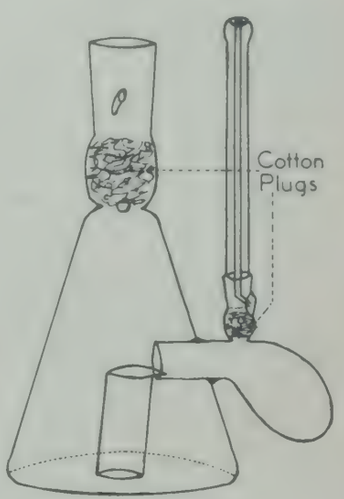


Fig. 27. Flasks used in Nitrogen Fixation Studies which permit the tipping of the contents of the center well into the sidearm; the inclined center well facilitates pouring.

Burk (1934) demonstrated that nitrogen fixation could be followed indirectly by estimating the growth rate of nitrogen fixing organisms as measured by oxygen uptake. In a nitrogen free medium the growth of azotobacter is limited by its ability to fix nitrogen. The more rapidly the organism fixes nitrogen the more rapidly it multiplies and in turn the more rapidly it takes up oxygen. Thus a measurement of the increasing rate of oxygen uptake gives a close approximation of the rate of nitrogen fixation although the two reactions are not strictly parallel.

MISCELLANEOUS METHODS

W. W. Umbreit

Vogler method: Vogler (1942) described a method for studying CO_2 fixation. This method is essentially that of Dickens and Simer (1933) except that Dixon-Keilin flasks were used and, at appropriate intervals, gases were added. The amounts of gas (CO_2) added were measured manometrically by introducing them into the sidearm and measuring the increased pressure resulting from such addition. A few moments are required before gases added to the sidearm diffuse into the main compartment where they react and this time is sufficiently long to permit accurate estimation of the amounts added. Under acid conditions Vogler was able to estimate CO_2 fixation and O_2 uptake simultaneously.

Gaffron method: In measurements of simultaneous oxidation of hydrogen and reduction of carbon dioxide, Gaffron (1942) has described in detail two methods for absorbing carbon dioxide after the reactions were complete. One of these employed flasks in which the sidearm is attached, not to the body of the flask itself, but to the ground glass joint by which the flask is attached to the manometer. The manometer connection itself has been partially cut away so that by rotating the flask in one direction, the sidearm becomes part of the gas space of the flask, whereas by a further rotation this arm may be closed off (see Fig. 28). There are some minor technical difficulties associated with the use of this type of system. For example, when the sidearm is closed off, pressure changes in the flask have no effect on this isolated gas space, hence upon connecting the sidearm with the main compartment a sudden equalization of pressure occurs between them. Yet this method is one of the few systems available in which one can successively expose tissues to CO_2- free gas and to gas with CO_2 (or other gases for which specific adsorbants are available).

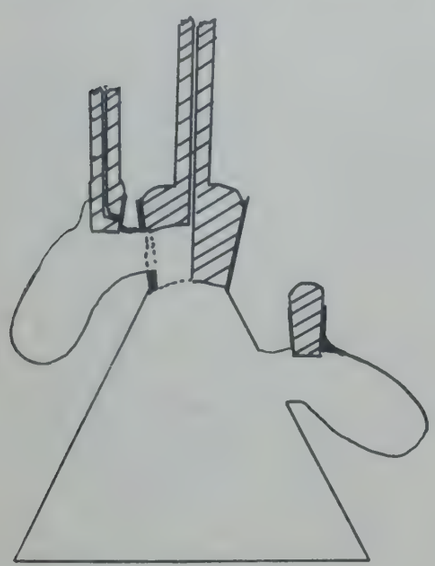


Fig. 28. Flask employed in the Gaffron Method.

The second method employs the usual type of flask with a glass paddle fused to the plug of the sidearm. In this sidearm is placed a small thin-walled glass bulb filled with KOH solution. Upon turning the plug of the sidearm the paddle is rotated so that it crushes the bulb, thus releasing the KOH which absorbs the CO_2.

Gas analysis: Two general types of procedure are employed in using the Warburg instrument as an instrument of gas analysis. For gas mixtures containing less than 2 to 2.5% of the gas to be measured, the flasks are filled with the gas to be analyzed (either by the flow method or the evacuation method, see Chapter 5). A reaction is then caused in the flask (or a reagent added) which will absorb the gas. The decreased pressure observed is a measure of the gas content. This method has been used for oxygen (see page 117) by using slightly acidified solutions of pyrogallol in the main compartment and tipping in alkali from the sidearm after the system is equilibrated. It has been used for CO_2 by generating alkali by means of permanganate and iodide (see page 112) or by using Dixon-Keilin flasks (e.g., see Vogler, 1942).

The second procedure, for gas mixtures containing more than 2 to 2.5% of the gas to be measured, is as follows: the reagent for absorbing the gas is placed in the main compartment of the flask and the flask filled with an inert gas. After equilibrium is obtained, the gas to be analyzed is introduced into the sidearm. The increased pressure, while the gas remains in the sidearm, is a measure of the total amount of gas introduced. As the gas diffuses into the main compartment the reagent absorbs it and the resulting decrease in pressure is a measure of the specific gas involved. Three methods of adding the gas to be analyzed may be used, as follows:

1. Addition to sidearm: Sidearm flasks equipped with a gas vent are employed (fig. 11, page 43). Before adding the reagent to the flask, the gas is allowed to flow through the sidearm plug until all the original gas in it has been displaced by the mixture to be analyzed. The reagent is added to the flask, the sidearm plug inserted (closed, so that there is no flow of the gas into the flask, reservoir remains attached to plug), and the gas in the flask is replaced with an inert mixture by evacuation procedure (see page 44). After equilibration (during which time the reagent absorbs any of the gas in question which may have entered the flask while inserting the sidearm plug), the plug is turned momentarily to allow gas to enter the flask. The increase in pressure is measured (giving the µl. of gas added). After the gas has diffused from the sidearm and been absorbed by the reagent, the pressure change may be used to calculate the composition of the gas added.
2. Addition to sidearm: This is essentially the same as above, except that the sidearm plug is filled with the gas mixture in question by evacuating and refilling with the gas mixture. Several (4 or 5) evacuations and refillings are necessary.
3. Addition through manometer stopcock: Gas may also be added through the stopcock of the manometer after flushing out the connecting tubing through the tail-vent

of the three-way stopcock. This is an especially convenient method of gas addition. However, the gas tends to remain in the capillaries for a long time. It may be forced into the flask by closing off the open end of the manometer and raising the fluid until it travels along the capillary connecting the manometer to the flask, but even then there is a "dead" space between this connection and the stop-cock. This method is useful when great accuracy is not required.

The reagents one employs for absorbing gases, depend upon the nature of the gas. Standard works on gas analysis should be consulted for their preparation.

Disinfectants and Germicides: The action of killing agents may be studied manometrically. In principle these methods depend upon adding the toxic agent to the metabolizing tissue or bacterial suspension and determining the amount of inhibition (of respiration or glycolysis) under specified conditions. See Bronfenbrenner, et al. (1938) and Ely (1939) for details.

OTHER TYPES OF RESPIROMETERS

W. W. Umbreit

Two types of constant-volume differential manometers have been devised which combine the advantages of the "Warburg" constant volume type and the "Barcroft" differential type. The first of these, the Dickens and Greville (1933) instrument is illustrated diagrammatically in Fig. 29. The volume is maintained at a constant value by adjusting the height of the manometer arms so that the fluid in the manometer remains at a constant mark. The difference in fluid levels in the two arms is read from a graduated scale. This permits one to employ a compensated system, independent of changes in barometric pressure, yet using the simple flask constants of the Warburg instrument. The second instrument, that of Summerson (1939) is illustrated in Fig. 30. It is an extremely adaptable instrument, being capable of serving as two ordinary Warburg manometers (which indeed comprise the main portion of the apparatus) or of serving as a constant volume differential manometer (in which case the two outer columns, attached to the flasks, are held constant and the difference in reading between the two inner columns noted. There is little doubt but what this is a very useful instrument, but at present it is not widely distributed.

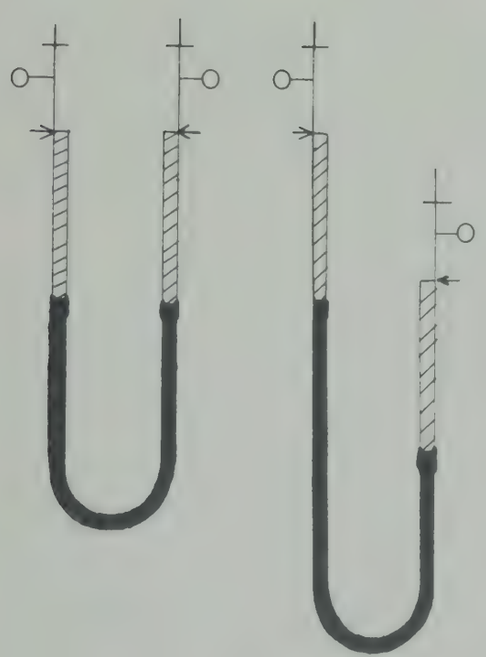


Fig. 29. Diagram of the principles of the Dickens-Greville Constant-Volume Differential Manometer.

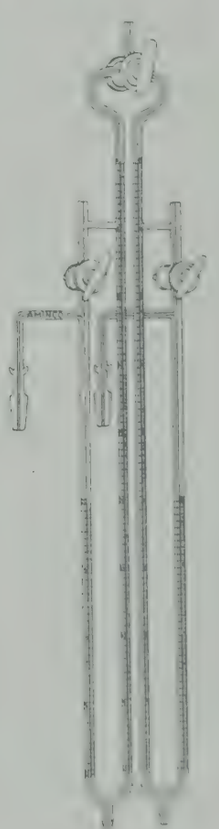


Fig. 30. Glassware for the Summerson Manometer.

For measuring smaller changes than the standard Warburg instrument is capable of accomplishing, several types of micro and ultra-micro manometric instruments have been developed. These are reviewed and described in some detail by Dixon (1943), Perkins (1943) and Tobias (1943) so that we will omit further consideration of them here.

Sometimes it is desirable to measure larger quantities of gas exchange than can be handled with the standard Warburg instrument. Macrorespirometers involving essentially the same principles but employing larger flasks may be used. Suitable modifications have been described by Wells (1938) and Wood, et al. (1940).

MEASUREMENT OF GROWTH RATES

R. H. Burris

In general bacterial enzymes are studied with resting cells or cell-free preparations as described in Chapter 8. However, it is desirable at times to follow the metabolism of growing microorganisms. With proliferating cells respiration is no longer linear with time but logarithmic, i.e., a plot of the logarithm of activity per unit time against time will yield a straight line in the ideal case. It cannot be assumed that every measure of activity, oxygen uptake, CO_2 output, methylene blue reducing power, etc., will exhibit a logarithmic increase with time for every organism. The validity of the assumed relationship must be established by concomitant estimation of increasing cell numbers, cell mass, or cell nitrogen. In studying growth processes, cells in their logarithmic growth phase should be used to avoid the complications introduced by the lag phase or phase of negative acceleration.

Burk (1934) reviewed a considerable amount of his experimental work with growing cultures of azotobacter. He demonstrated that in cultures of azotobacter fixing molecular nitrogen the rate of respiration was a measure of growth, and that the growth rate was determined by the rate of nitrogen fixation. Although the relationship was not absolutely quantitative, measurements of oxygen uptake still constituted an excellent measure of growth or nitrogen fixation. He expressed the velocity of growth or nitrogen fixation as the first order velocity constant g (Wilson, 1940, has preferred to substitute k for g to avoid confusion with g as an expression of generation time) where

$$(53) \quad g = k = \frac{2.303\, d \log (a + y)}{dt}$$

$$= \frac{dy}{(a + y)\, dt}$$

a is the initial concentration of azotobacter and y is the increase in t hours. Thus, k is related to the generation time, or number of hours required for doubling the cell concentration, by

$$(54) \quad k = \frac{2.303 \log 2}{\text{generation time}} = \frac{0.695}{\text{generation time}}$$

By plotting the logarithm of the respiration rate for unit intervals against time, k can be evaluated as the slope of the resulting line times 2.303. The k value as a measure of rate is much more valuable than a mere measurement of initial and final numbers or concentrations, for these latter evaluations are subject to unnoticed shifts in rate during the course of the reaction.

The following will serve to illustrate the manner of setting up, observing, and plotting an experiment with a growing culture. To 40 ml. of nitrogen-free medium 4 drops of a 24-hour culture of Azotobacter vinelandii was added, the inoculated medium was shaken vigorously, and 2 ml. was added to each Warburg flask. Since the rate of respiration increases with time it is necessary to start with light suspensions of the organisms; an initial uptake of 20 to 70 µl. oxygen per hour per flask is suitable. The gas mixtures were added to the flasks by the evacuation procedure outlined in Chapter 5. After bringing the flasks to temperature equilibrium at 31°C., the manometers were closed and readings taken at 30-minute intervals thereafter. In table XX we have listed the µl. uptake for each 60-minute period as recorded for duplicate flasks; only two gas mixtures of the experiment are shown.

TABLE XX

Hydrogen Inhibition of a Growing Culture of Azotobacter vinelandii

Gas Mixture	Flask	0-60 min. µl. O_2 uptake	0-60 min. log µl. O_2 uptake	30-90 min. µl. O_2 uptake	30-90 min. log µl. O_2 uptake	60-120 min. µl. O_2 uptake	60-120 min. log µl. O_2 uptake	90-150 min. µl. O_2 uptake	90-150 min. log µl. O_2 uptake	120-180 min. µl. O_2 uptake	120-180 min. log µl. O_2 uptake
60 H_2, 20 N_2, 20 O_2.	1	63.8	1.805	68.8	1.838	70.5	1.848	74.2	1.871	81.7	1.913
	2	63.0	1.800	68.2	1.834	72.5	1.860	77.7	1.891	79.2	1.899
60 He, 20 N_2, 20 O_2.	3	64.4	1.809	70.5	1.848	77.0	1.887	88.9	1.949	97.2	1.988
	4	63.2	1.801	69.8	1.844	77.8	1.891	86.8	1.938	94.0	1.973

		150-210 min.		180-240 min.		210-270 min.		240-300 min.	
	1	88.5	1.947	94.8	1.977	100.7	2.002	106.9	2.029
	2	85.6	1.933	96.0	1.982	103.5	2.015	110.2	2.043
	3	109.3	2.039	124.5	2.096	137.5	2.138	153.2	2.185
	4	105.6	2.024	123.0	2.090	139.6	2.145	155.8	2.192

The data from table XX are plotted in Fig. 31 with time as abscissa and with oxygen uptake per hour and logarithm of oxygen uptake per hour as ordinates in parts I and II respectively. Plot I indicates increasing rates of respiration by the increasing slopes of the curves with time. Plot II is linear; the straight line through the points is best calculated by the method of least squares. The slope of the line times 2.303 gives the k value of the culture; from Fig. 31, II, the k values are 0.219 for A and 0.127 for B. As mentioned, the generation time equals 0.695/k, which gives 3.17 hours for A and 5.47 hours for B.

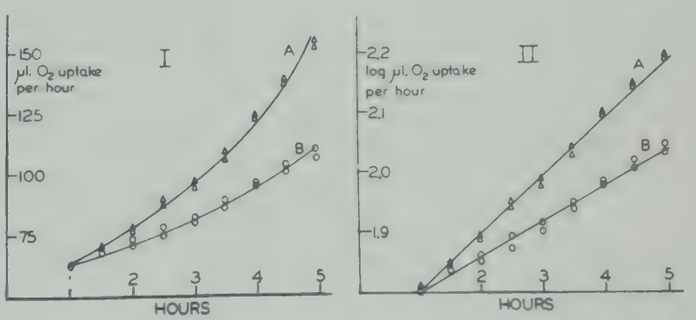


Fig. 31. Rate and log rate plots of oxygen uptake by a growing culture of Azotobacter vinelandii.

In the experiment described the nitrogen-free medium limited the growth of organisms other than azotobacter. In experiments lasting for periods greater than 4 to 5 hours and employing media subject to ready contamination, respirometer vessels should be steamed before use to reduce the load of contaminants. When it is necessary to follow activity for a day or more it is essential that the flasks and all material added to them be sterilized. Under such conditions special flasks, Fig. 32, designed to retain a cotton plug must be employed. A venting sidearm with cotton plug retainer may be attached for the addition of solutions and for flushing gases through the system, or a solid sidearm will serve if gases are changed by evacuation.

When using these flasks, sterilize them with the indicated cotton plug in position and another cotton plug in the top. When pipetting the culture into the flask, discard the top plug and remove and replace the lower plug with sterilized forceps. Do not sterilize the medium (or KOH) in the flask; much more uniform results will be obtained if a bottle of medium is inoculated, shaken and then pipetted aseptically into the respirometer vessels.

DETERMINATION OF THE TYPE OF INHIBITION

R. H. Burris

In interpreting the mechanism of enzyme action it is frequently very helpful to understand the manner in which specific inhibitors function. The usual basic distinction is that between competitive and non-competitive inhibitors. In the case of competitive inhibition the substrate and inhibitor are competing for enzyme surface, and hence changes in either inhibitor or substrate concentration will affect the degree of inhibition observed. Substrate concentration is without influence in non-competitive inhibition.

The mere observation of the constancy of inhibition at one inhibitor concentration and with varying substrate concentration should not be relied upon to establish the competitive or non-competitive nature of the inhibition. The more reliable method is to apply the equations for testing the type of inhibition that have been described by Lineweaver and Burk (1934). Derivation of the equations in detail has been given by Wilson (1939). Ebersole, Guttentag and Wilson (1944) have discussed competitive, non-competitive and other less common types of inhibition, suggested by Burk. These references should be consulted for adequate derivation of the theory upon which the tests for type of inhibition are based.

The application of the tests is relatively easy. Determine the velocity constants, v (the k value discussed under "Measurement of Growth Rates" is an example of a velocity constant), of the reaction over as wide a range of substrate concentrations as is practical and at two or more concentrations of inhibitor. As pointed out by Ebersole, Guttentag, and Wilson (1944), if the reciprocal of the velocity constant, $1/v$, is plotted against the reciprocal of the concentration of substrate, $1/(S)$, straight lines (calculated by the method of least squares) should result with the following characteristics:

I. In the absence of inhibitor, a straight line results whose slope/intercept equals K_s, the dissociation constant of the enzyme-substrate complex.

II. In strictly competitive inhibition, the intercept remains constant, but the slope is increased by $(1 + (I)/K_i)$, where (I) is concentration of inhibitor, and K_i the dissociation constant of the enzyme-inhibitor complex. The apparent K_s = slope/intercept increased by the same factor, i.e., $(1 + (I)/K_i)$.

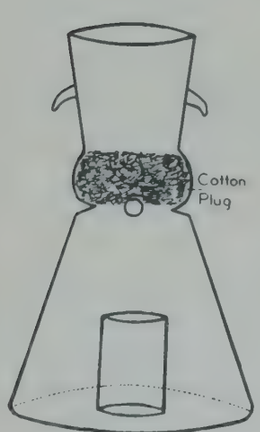


Fig. 32. Respiration vessel with cotton plug for maintaining aseptic conditions.

III. In strictly non-competitive inhibition, both the slope and intercept are increased by the same quantity, viz., $(1 + (I)/K_i)$ so that K_s remains constant.

Figure 33, from Wilson (1939), shows a plot of the reciprocal of substrate concentration, $1/pN_2$, against the reciprocal of the velocity constant, $1/k$, at varying inhibitor concentrations, H_2 pressures of 0.2, 0.4 and 0.6 atmospheres. The intercepts are common, but the slopes increase with increasing inhibitor concentrations indicating that H_2 acts as a competitive inhibitor in the symbiotic nitrogen fixation system of red clover. Figure 34, from Ebersole, Guttentag and Wilson (1944), shows an example of non-competitive inhibition, namely the CO inhibition of non-symbiotic nitrogen fixation by Azotobacter vinelandii. In this case the intercepts and slopes both increase with increasing concentrations of the inhibitor, CO.

If the data do not fit the described conditions the inhibition may be of the "uncompetitive" or "quadratic" type. Consult Ebersole, Guttentag and Wilson (1944) for a description of tests for determining inhibition of such nature.

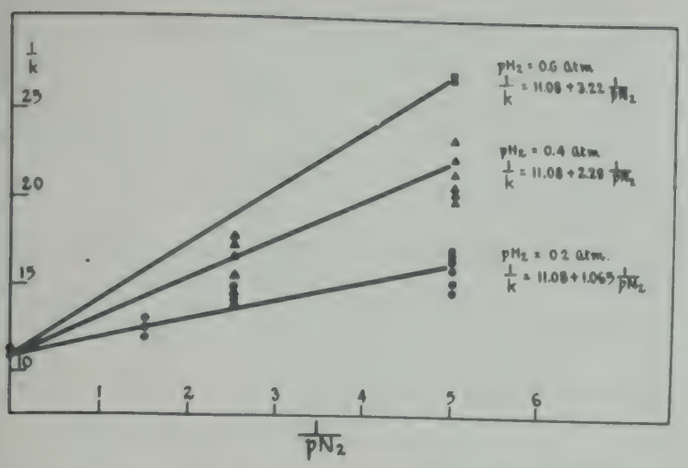


Fig. 33. Hydrogen as a competitive inhibitor of nitrogen fixation by red clover.

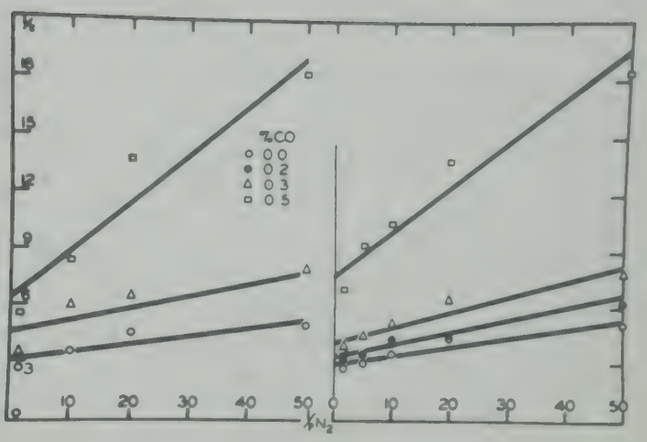


Fig. 34. Carbon monoxide as a non-competitive inhibitor of nitrogen fixation by Azotobacter vinelandii.

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Chapter Twelve

"THUNBERG" TECHNIQUES FOR ESTIMATION OF DEHYDROGENASE ACTIVITY

R. H. Burris

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Chapter Twelve

"THUNBERG" TECHNIQUES FOR ESTIMATION OF DEHYDROGENASE ACTIVITY

R. H. Burris

It is recognized that the uptake of oxygen and the liberation of CO_2 are in reality only the end reactions of a relatively long series of oxidation-reduction reactions. Frequently it is desirable to study these intermediate reactions by means of the "methylene blue" or "Thunberg" technique, which is here described.

THE THUNBERG METHOD

In this method tubes as shown in Fig. 35 are employed. These are provided with a sidearm cap arranged to hold materials to be added to the main tube after evacuation. Air is withdrawn through the evacuation outlet when the sidearm cap is turned so that a hole drilled in its standard taper inner joint coincides with the evacuation outlet. The tube may be closed off after evacuation by turning the sidearm.

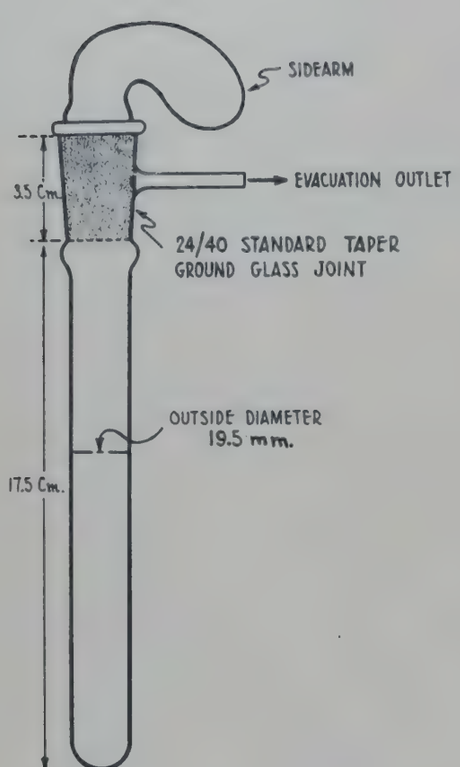

Fig. 35. Thunberg tube.

Distribution and concentrations of reacting components may vary with the application of the method, but the following directions constitute average conditions employed in measurements of methylene blue reduction.

Place 1 ml. 1/10,000 methylene blue, 2 ml. M/50 substrate and 2 ml. M/15 phosphate buffer (pH 7.0) in the tube and 1 ml. of bacterial suspension or tissue suspension in the sidearm cap. Put 2 lines of anhydrous lanolin or other grease on the ground joint of the cap at 90° from the hole in the ground joint. Connect the cap and tube with the hole in the cap opposite the evacuation outlet on the tube joint. Press the cap firmly in place so the grease from the two lines flows over the entire joint; do not rotate the joint more than a few degrees. By setting the joint in this manner air is not trapped in the grease. Attach the side outlet of the tube to a good water aspirator or a vacuum oil pump, and evacuate for 3 minutes, tapping the tube to release dissolved gas; keep the tube inclined at a low angle to minimize "bumping". A water aspirator is entirely satisfactory for evacuating Thunberg tubes. When evacuation is complete, rotate the cap slowly through an angle of 180°, and then oscillate it through a small arc to set the cap. Put the tube in a constant temperature water bath, and after allowing 10 minutes to reach temperature equilibrium, invert the tube to mix the contents, and follow the methylene blue reduction visually or photometrically. For visual measurement a tube is included which contains all the components of the system being studied (the active tissue is poisoned or heat inactivated) but with the methylene blue at 1/10 normal concentration. This tube represents 90% reduction of the methylene blue, and when the other tubes match the color intensity of this tube the time is recorded as the end point.

The reliability of the results obtained by the Thunberg method depends in part upon the efficiency with which oxygen is removed. Obviously, since leuco-methylene blue is converted into the blue form by oxygen, it will be impossible to measure the real reduction time if some of the dye is being reoxidized by oxygen during the process. It has been found in practice that adequate removal of oxygen is obtained with evacuation by means of an ordinary laboratory water-pump provided the evacuation is continued for at least three minutes and the tube is tapped vigorously during the evacuation. Fig. 36 from Tam (1939), indicates that methylene blue reduction occurs at approximately the same rate with a three minute evacuation as with evacuation plus an N_2 flush followed by a final evacuation.

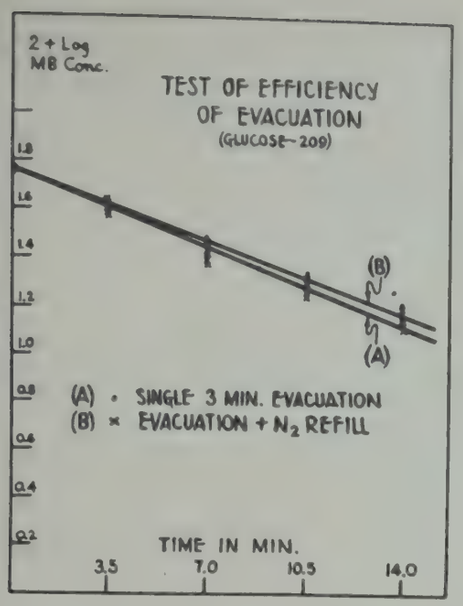

Fig. 36. Effect of evacuation procedure on methylene blue reduction by Rhizobium trifolii 209 on a glucose substrate.

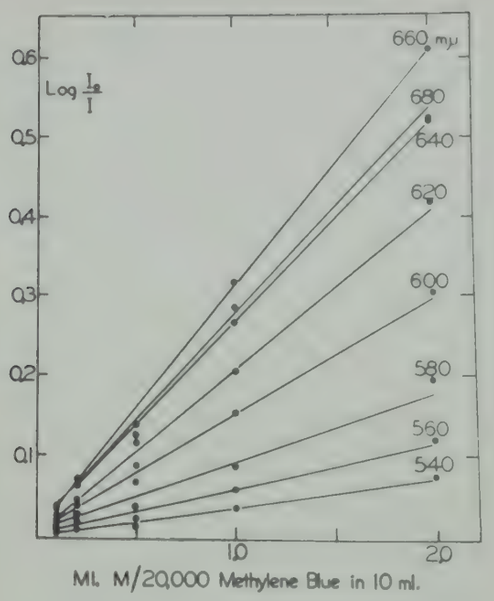

Fig. 37. Absorption of light of different wave lengths by methylene blue.

PHOTOMETRIC ESTIMATION OF METHYLENE BLUE REDUCTION

Methylene blue reduction is not a strictly linear function and much more information on the kinetics of the reaction can be obtained if the reduction is followed photometrically rather than visually. Tam and Wilson (1941) have described such a method using the Evelyn photometer; the tube shown in Fig. 35 is of proper dimensions for this application. The concentration of methylene blue and the total volume used depend upon the type of photometer employed. With the Evelyn photometer, Tam and Wilson (1941) added concentrations of methylene blue, substrate, buffer and tissue suspension as described in the preceding section. With the Coleman Universal spectrophotometer a lower concentration of methylene blue is desirable; 1 ml. M/20,000 in a final volume of 10 ml. is satisfactory. The 660 mμ wave band is used. Fig. 37 shows the light absorption of methylene blue at various wave lengths as determined with the Coleman Universal spectrophotometer; maximum absorption is at 660 mμ and the absorption is a reasonably linear function of methylene blue concentration at this wave length.

To follow methylene blue reduction photometrically a series of tubes are brought to temperature equilibrium, their contents mixed at 15 or 30 second intervals, the tubes wiped dry and initial readings taken immediately after each tube is mixed. The tube is returned to the bath and read every 3 minutes, thus with 15 second intervals 12 tubes can be read every 3 minutes. Suspension concentrations are adjusted so reduction time is from 15 to 30 minutes giving 5 to 10 readings to plot. It is unnecessary to follow the tubes to complete reduction. Upon finishing the experiment a few crystals of sodium hydrosulfite are added to each tube to completely reduce the methylene blue, and the tubes are then read on the photometer, (I_o). The concentration of methylene blue at any time is proportional to log (I_o/I), where I is the galvanometer reading at any particular time and I_o is the reading after complete reduction. If reduction is linear with time a plot of log (I_o/I) against time will yield a straight line. Tam and Wilson (1941) found that with most substrates the reduction was logarithmic rather than linear with respect to time, hence it was necessary to plot log of methylene blue concentration, i.e., log [log (I_o/I)], against time. The following data from Tam and Wilson (1941), (table XXI) graphed in Fig. 38, will serve to illustrate the manner in which readings are plotted.

In the experiment for which data are recorded in table XXI, galvanometer readings (I) were taken at 0, 3, 6, 9, and 12 minutes; end point galvanometer readings (I_o) for each tube were taken after the addition of sodium hydrosulfite. The values for log (I_o/I) are recorded in the table. The logs of these values were then taken. To each number, 2 was added to avoid plotting on a negative scale; this addition does not alter the slopes of the lines. By plotting time against 2 + log [log (I_o/I)] the straight lines of Fig. 38a are obtained; their slopes can be determined from the graph. In Fig. 38b the slopes

(rates of methylene blue reduction) are plotted against pH to give a pH-activity curve for methylene blue reduction by Rhizobium trifolii 209 on a succinate substrate.

TABLE XXI

Effect of pH on Rate of Methylene Blue Reduction by Rhizobium trifolii, Sodium Succinate Substrate

pH	Log (I_o/I)					2 + Log [Log (I_o/I)]					Slope of line from fig.38a
	0 min.	3	6	9	12	0 min.	3	6	9	12	
4.40	.542	.549	.549	.538	.523	1.734	1.739	1.739	1.731	1.719	.0012
5.25	.569	.523	.482	.453	.417	1.755	1.719	1.683	1.656	1.620	.0112
6.15	.594	.523	.459	.409	.369	1.774	1.719	1.662	1.612	1.567	.0172
6.50	.545	.469	.385	.328	.272	1.736	1.671	1.585	1.516	1.435	.0251
6.95	.561	.456	.367	.310	.254	1.749	1.659	1.565	1.491	1.405	.0287
7.20	.569	.438	.349	.282	.213	1.755	1.641	1.544	1.450	1.328	.0356
7.47	.602	.435	.305	.231	.166	1.780	1.638	1.484	1.364	1.220	.0467
7.83	.594	.429	.301	.197	.128	1.774	1.632	1.479	1.294	1.107	.0556
8.20	.602	.444	.301	.186	.122	1.780	1.647	1.479	1.269	1.086	.0578
8.88	.594	.469	.342	.235	.158	1.774	1.671	1.534	1.371	1.199	.0400

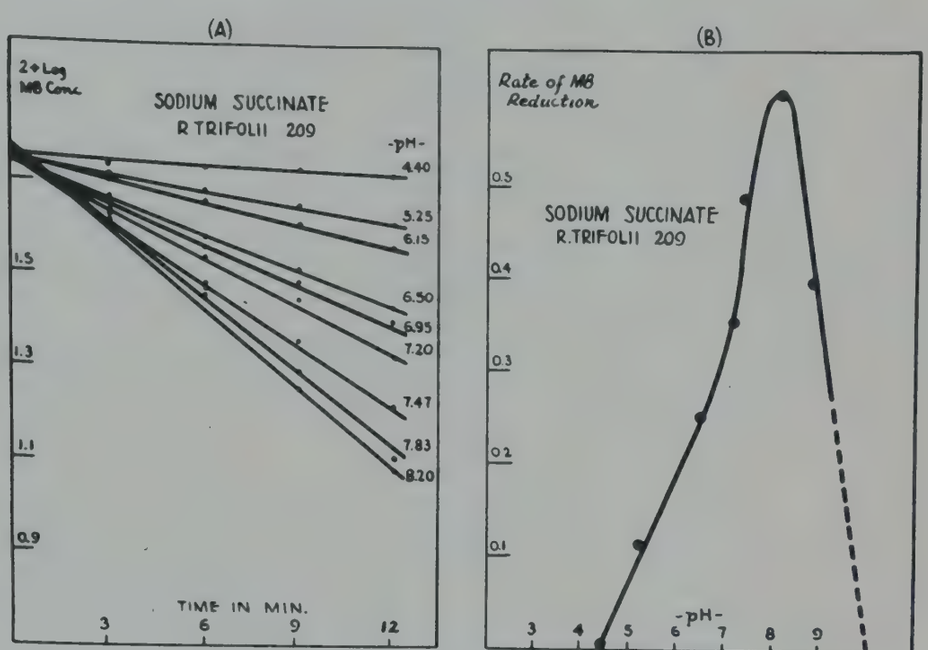

Fig. 38. Methylene blue reduction by Rhizobium trifolii 209 as affected by pH. Sodium succinate substrate.

It is of interest to point out the wide variety of studies which can be made with a technique of this sort. Tam and Wilson (1941), for example, determined pH optima of several substrates, the temperature relationships which permitted the calculation of energy of activation, the comparative dehydrogenation of a wide variety of substrates, and the effect of a variety of inhibitors.

SIMPLIFIED METHODS

When it is necessary to make many simultaneous estimations of methylene blue reduction time, the number of Thunberg tubes available may restrict the observations. If limited accuracy will suffice, a simplified technique using ordinary test tubes without evacuation may be employed. Such a procedure has been described by Friedeman and Hollander (1942). The following is the modification sometimes used in our laboratory on bacterial suspensions:

To an ordinary test tube add 0.5 ml. of substrate and 0.5 ml. of 1/4000 methylene blue. Add 2 ml. of 2% agar, in 0.5% K_2HPO_4 adjusted to pH 7.0, which has been melted and cooled to 45° C. Add 1 ml. of the cell suspension, mix, and chill in an ice bath until solidified (1 to 2 minutes). Place tubes in a water bath at 37° to 40° C. and determine the time required for reduction. A blue zone (2 to 3 mm.) at the top of the tube results from the diffusion of oxygen from the air and offers a sharp contrast to the reduced portion below.

OTHER RELATIONSHIPS

Methods for measuring methylene blue reduction other than those described but employing electric photometers have been described especially by Ganapathy and Sastri (1938) and by Jongbloed (1938). Methylene blue and other dyes sometimes exert a toxic effect on the tissues; Quastel and Wheatley (1931), Yudkin (1933), and Tam and Wilson (1941) report that free phosphates protect against this effect, hence the usual methods employ buffers high in phosphate. As is true of many other cases the method of growing and treating the tissue to be studied may have a marked influence on the rates of dehydrogenation obtained; an example is shown by the work of Wood and Gunsalus (1942).

At times methylene blue (or other dye system of proper potential) may be used to "by pass" a normal system in the cell; for example, its use in restoring the respiration of red blood cells poisoned with cyanide (Barron and Harrop, 1928) has become classic. In some reconstructed enzyme systems methylene blue may be used as an actual carrier of the hydrogen.

By establishing anaerobic conditions with an atmosphere of hydrogen in Warburg vessels Wilson, Lee and Wilson (1942) and Wilson, Burris and Coffee (1943) followed hydrogen uptake by hydrogenase preparations when methylene blue was supplied as hydrogen acceptor. It was necessary to use high concentrations of methylene blue, as the reduction of 1 ml. of a M/1,000 solution requires only 22.4 µl. of hydrogen.

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Chapter Thirteen

RELATED ELECTROMETRIC TECHNIQUES

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Chapter Thirteen

RELATED ELECTROMETRIC TECHNIQUES

INTRODUCTION

Potentimetric techniques widely used in the study of tissue metabolism involve the measurement of pH (by glass electrode or quinhydrone electrode methods), the measurement of oxidation reduction potentials, and the measurement of oxygen utilization or production by means of the dropping mercury electrode. Since detailed instructions of the use of the glass electrode usually accompany the instrument, it is not discussed here. The other two methods are less well known.

THE DETERMINATION OF DISSOLVED OXYGEN BY MEANS OF THE DROPPING MERCURY ELECTRODE

J. F. Stauffer

As ordinarily referred to, the dropping mercury electrode is but a portion of the elaborate self-recording instrument known as the polarograph. This instrument plots the current-voltage curve of electrolysis of a solution contained in an electrolysis cell, of which the dropping mercury electrode is the cathode and a pool of mercury is the anode (in certain instances the polarity of the cell is reversed). After standardization of the instrument, the current-voltage curve can be interpreted to determine the substances (of a wide variety), and the concentration of each present in the solution. The unique portion of the polarograph is the dropping mercury electrode; the remainder of the instrument consists of the necessary accessories for applying a constantly increasing voltage, for measuring the current, and for recording the current-voltage data as a continuous curve.

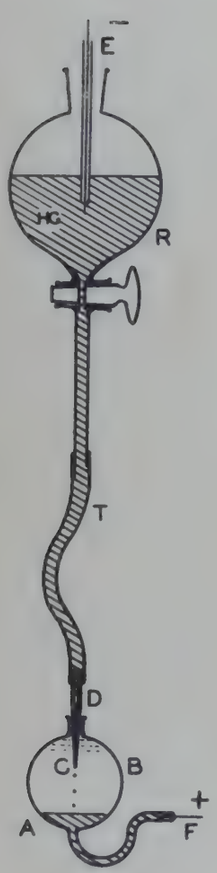

Fig. 39. The dropping mercury electrode.

One type of dropping mercury electrode is represented in Fig. 39. The electrolysis cell, B, contains the solution to be electrolyzed and a relatively large pool of mercury, A, which serves as the anode. The dropping mercury electrode, C, consists of small drops of mercury constantly forming and dropping from the tip of the small-diameter glass capillary tube, D. The reserve of mercury is contained in the separatory funnel, R, and delivered at a constant head (pressure of Hg) through a piece of pure gum rubber tubing, T, to the capillary, D. This arrangement insures a constant drop rate; the rate depends on the particular instrument used and upon the experimental conditions, etc., but the usual rate is one drop every one to one and a half seconds. The mercury pool, A, remains at the same height by discharging the mercury added to it through the overflow tube, O. The dropping mercury electrode assembly is provided with the platinum electrode E, and the platinum wire, F, protruding from the overflow tube for electrical connection to the circuit of the polarograph.

A diagrammatic representation of a current-voltage curve, such as one might obtain with the polarograph in the electrolysis of a dilute (10^{-4} to 10^{-2} M) solution containing two metallic ions, is shown in Fig. 40. Two abrupt vertical displacements of this curve are shown at A and B, and as the voltage is increased the current increases at a much increased rate for a relatively small change in voltage up to a point where its change approximates the original nearly horizontal portion of the curve. Each region of the vertical displacement of the curve, A and B, represents the voltage where the ions of one of the substances is being completely reduced (or is being discharged) at the dropping mercury electrode (the cathode). The position of A and B referred to the voltage axis gives the "decomposition voltages" E_1 and E_2 of the two substances. Since this curve is reproducible, insofar as the position along the voltage axis where the vertical displacements occur, E_1 and E_2 serve to identify the two particular substances present. During the discharge of an ion, the current becomes directly proportional to the applied voltage as long as the supply of ions to the electrode (in this case the cathode) is not limiting.

Finally, however, a voltage is reached where the supply of reducible ions becomes limiting; the current-voltage curve then levels off (it is not exactly horizontal because of the small residual current). As the number of ions of a reducible substance diffusing to the electrode determine the current carried once the decomposition voltage has been reached, it is readily understood that the displacement of the current-voltage curve along the current axis is a measure of the concentration of the particular substance being reduced. Then the vertical distances, C_1 and C_2 respectively represent the concentration of the two substances in the solution, in this case the two metallic ions. Since, at the concentrations employed, ionization is practically complete, C_1 and C_2 represent the molar concentration of the two ions.

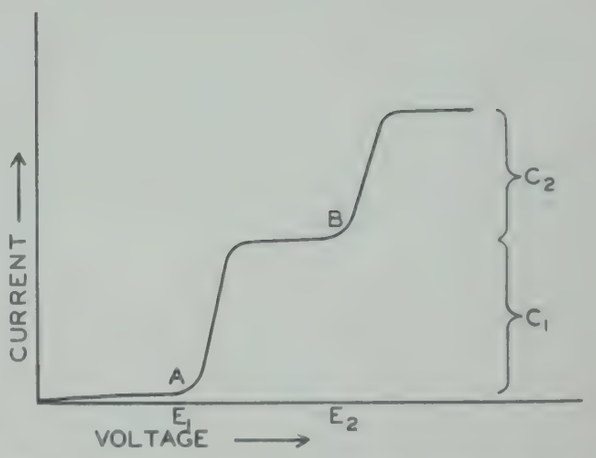

Fig. 40. Current-voltage curve of a solution containing two metallic ions.

From the above brief discussion of the principle of the polarograph, it is apparent that the instrument affords a means of rapid quantitative and qualitative analysis (it has many other applications which will not be discussed here, but see Kolthoff and Lingane (1941), and the literature listed in brochures describing the polarograph from apparatus supply companies). The points we wish to emphasize are:

1) Each particular substance which reacts has a decomposition voltage,
2) The difference in the amount of current at the decomposition voltage and at the voltage where the current is limited by the supply of reducible substance is a measure of the concentration of the substance.

On this basis it is possible to effect a simplification of the apparatus required to determine the concentration of one particular substance, in this case dissolved oxygen in biological systems (Petering and Daniels, 1938).

It would appear that if one were analyzing a solution for a single substance, a determination of the difference in current flowing at an applied voltage slightly less than the decomposition voltage of the substance and one in the region of the limiting current would

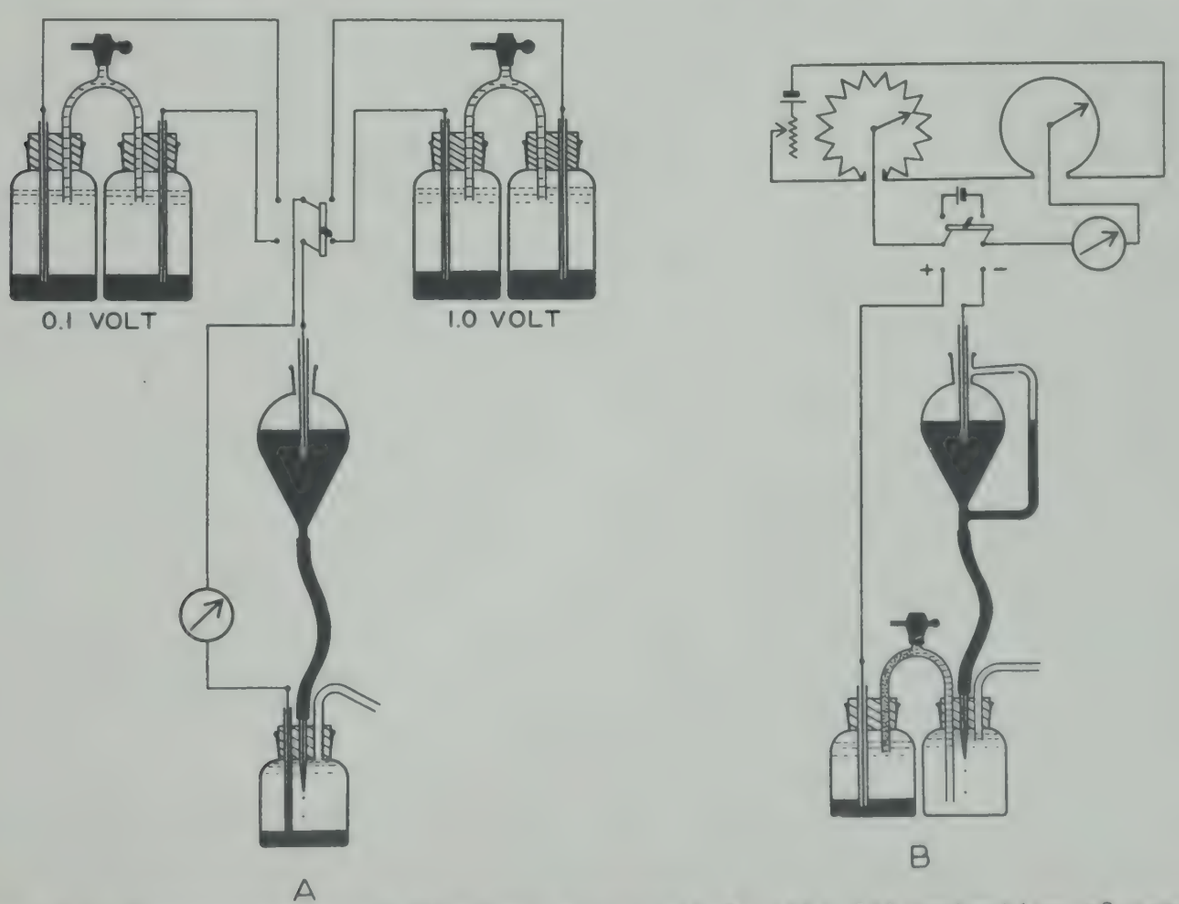

Fig. 41. Dropping mercury electrode arranged for the determination of oxygen.

be sufficient to determine the concentration of the substance; furthermore, changes in concentration of the substance could be followed by successive measurement of this "current difference". That the above is true for dissolved oxygen, has been shown by Petering and Daniels (1938). As they point out, since only two voltages (0.1 and 1.0) are necessary for the analysis, the apparatus set-up is very simple, i.e., the dropping mercury electrode assembly (Fig. 39) is connected into a circuit having a galvanometer and either a potentiometer or two large capacity standard cells. These types of the circuit are diagrammed in Fig. 41.

The dropping mercury electrode is easily assembled. A separatory funnel or a leveling bulb serves to contain the supply of chemically pure mercury (the mercury may be reclaimed by cleaning it with dilute (1 to 10) nitric acid, followed by distillation), which is supplied to the glass capillary through a piece of pure gum rubber tubing or rubber tubing that has been soaked in hot concentrated alkali to free it of sulfur. The glass capillary which has a fine tip (ca. 0.5 mm. O. D.) with the capillary reduced to such a size that the drop rate is one drop every one to one and one-half seconds with a 30-45 cm. "head" of mercury, may be prepared by drawing down a piece of glass capillary of a broken laboratory thermometer. The electrolysis cell may be an ordinary gas bottle fitted with a rubber stopper bearing the glass capillary, an overflow tube and a sealed in platinum electrode, which is submerged in the pool of mercury at the bottom of the bottle (Fig. 41a), or a cell provided with parallel glass (or quartz) windows may be used. The latter, which is particularly adapted to experiments involving irradiation of the cell contents, is equipped with an overflow tube for the mercury and a constricted neck in which the tapering portion of the glass capillary rests, or is ground in to form a firm joint. If the solution to be placed in the electrolysis cell contains cells or enzymes that are very sensitive to mercury compounds, the anodic pool of mercury may be placed in a separate cell which contains saturated KCl-calomel and is connected to the dropping mercury electrode vessel by means of a saturated KCl-agar bridge (fig. 41b).

Any available type of potentiometer, a "student type" for example, may be used to supply the two potentials (0.1 and 1.0 volts). It is essential that the potentiometer be frequently standardized during an experiment with a standard cell for slight changes in applied voltage lead to erratic "current differences". As indicated above, the two potentials may be obtained from two standard cells; their preparation is described by Petering and Daniels (1938).

An ordinary type galvanometer with a 10 cm. scale or the standard wall reflecting galvanometer will serve to measure the current; one having a sensitivity of 5×10^{-7} ampere per scale division is sufficient (Petering and Daniels, 1938). We have successfully used an inexpensive galvanometer with lamp and scale (sensitivity, 0.025 microampere; period, 3 seconds). The ground glass scale was replaced with a curved transparent centimeter ruler whose back had been rubbed with fine emery paper to increase the sharpness of the image of the lamp filament. The galvanometer sensitivity may be reduced by use of a suitable shunt.

The dropping mercury electrode is calibrated against known concentrations of oxygen by approximately simultaneously determined current differences and oxygen concentrations; oxygen is determined by the Winkler method.

But before giving the details of the method of calibration, it will not be amiss to point out that the following conditions must be adhered to in order that the calibration may be used to determine the oxygen concentration during an experiment and from one experiment to the next:

1) The solution must be of the same composition.
2) The mercury pressure (i.e., the distance from the tip of the capillary to the surface of the mercury in the reservoir) must be maintained constant.
3) The same capillary must be used.
4) All determinations should be made at the same temperature.

The calibration is as follows: prepare about five liters of nutrient solution, or buffer and add to it the cells or preparation which will be used in the experiments (only a small quantity need be added) or simply pass the liquid through filter paper. This is necessary to eliminate or suppress the "absorption maximum" in the current-voltage curve

(c.f., Petering and Daniels, 1938; Koltoff and Lingane, 1941). Distribute the solution in five liter flasks, each of which is equipped with a rubber stopper bearing a siphon and a gas intake and outlet tube. Saturate the solution in one flask with air at the temperature of calibration, by passing it in through the siphon. The solution is now siphoned out of the flask into the electrolysis cell. Allow the solution to be displaced several times with the tip of the siphon being held near the bottom of the cell. Insert the glass capillary, and proceed to determine the galvanometer deflection at 0.1 and 1.0 volt respectively. Fill two 250 ml. glass stoppered bottles, whose volume is exactly known, with the solution, and analyze for oxygen immediately by the Winkler method (Treadwell and Hall, 1937). Reduce the oxygen content of the solution in one of the remaining flasks by passing in nitrogen (air with its oxygen content reduced by passage through alkaline pyrogallic acid may be used instead) for a few minutes and vigorously shaking the flask. Determine the current difference and the oxygen content as before. Repeat these determinations with successively lower concentrations of oxygen in the remaining lots of solution. The current differences are now plotted against oxygen concentrations to obtain the calibration curve. The points should fall on or very close to a straight line. This calibration curve may now be used to determine the oxygen concentration, or to follow changes in oxygen concentration when the electrolysis cell contains the solution plus the experimental material.

As to the change in oxygen concentration which can be measured by this method, Petering and Daniels (1938) show that when an electrolysis cell containing about 10 ml. is used a total change in oxygen concentration of the order of 5×10^{-9} mole can be determined; this corresponds to 0.112 µl. and to 0.016 p.p.m. of oxygen at room temperature.

This method has been shown to be suited to the determination of the oxygen changes during respiration and photosynthesis of algal cell suspensions, during respiration of yeast, blood cells and animal tissues (Petering and Daniels, 1938). Some specific references of its use, are as follows: photosynthesis (Petering, Duggar and Daniels, 1939; Dutton and Manning, 1941; Weybrew, 1942); oxygen content of soils (Karsten, 1938); respiration of yeast (Anderson and Duggar, 1941).

THE DETERMINATION OF OXIDATION-REDUCTION POTENTIALS

A. M. Hanson

The theory and use of oxidation reduction potential measurements is adequately described by Axelrod and Johnson (1939) and in most text books of physical chemistry. It is useful, however, to briefly recall the relationships derived.

The theory is based upon the passage of electrons to and from an inert metal and a solution. Passing from the metal to the solution the electrons combine with H^+ ions forming $(1/2\ H_2)$, illustrated in equation (55):

increase H^+ conc.: electrode becomes more positive since electrons leave.

(55) $H^+ + e \rightleftharpoons 1/2\ H_2$

metal electrode

increase H_2 pressure: electrode becomes more negative since electrons go in.

This equation (55) is purely a chemical equilibrium, i.e., $\frac{(H^+)\ (e)}{\sqrt{H_2}} = K'$ but since the electron content of the solution is constant (at a very low value) because electrons escape or come back from the metal, the electrode equation (55) becomes:

$$(56)\quad K'/e = K = \frac{H^+}{\sqrt{H_2}}$$

It is known from thermodynamics that the maximum work that one can obtain from any reaction (the "free energy") is:

$-\Delta F$ = Work (maximum) = RT ln K
R = gas constant
T = absolute temperature
ln = natural logarithm
K = the equilibrium constant.

Hence in the case cited in equation (56):

$$-\Delta F = \text{Work (maximum)} = RT \ln \frac{H^+}{\sqrt{H_2}}$$

Work, of course, can be expressed in electrical units, i.e.,

$$\text{Work} = \underset{\text{(volts)}}{\text{potential}} \times \text{Faraday} = EF$$

Thus:

$$EF = RT \ln \frac{H^+}{\sqrt{H_2}} \quad \text{or}$$

$$E = \frac{RT}{F} \ln \frac{H^+}{\sqrt{H_2}} \quad \text{or}$$

$$E = \frac{RT}{F} \ln H^+ - \frac{RT}{2F} \ln (H_2), \text{ which is the fundamental electrode}$$

equation.

Or since $\ln A = 2.3 \log A$

$$E = 2.3 \frac{RT}{F} \log H^+ - \frac{2.3\ RT}{2F} \log (H_2)$$

Also the term $\log H^+$ is a function of pH. To obtain an expression relating the "hydrogen pressure" to the reducing or oxidizing properties of any system one may wish to measure, one can consider that in any aqueous solution, containing an activating enzyme, there exists a dynamic equilibrium between the oxidized and reduced forms of the compound being activated as follows:

$$(\text{Oxidized}) + H_2 \rightleftharpoons (\text{Reduced})$$

One must, of course, know something of the system one is measuring for the "Hydrogen pressure" depends upon the actual reactants of the system. The generalized equation as stated by Axelrod and Johnson (1939) is:

$$(58)\quad E = E_o + 2.3 \frac{RT}{nF} \log \frac{(Ox)}{(Red)} + \frac{(n-k)}{nF} 2.3\ RT \log H^+$$

Where: n = valence changes involved in the reaction
k = cations created by hydrogenation

$$E_o = -2.3 \frac{RT}{nF} \log K$$

Where K is the equilibrium constant of the reaction.

The potential determined on an inert electrode is thus a measure of the "hydrogen pressure" of a solution. This potential depends upon the system or systems involved (E_o), the ratio of the reduced to the oxidized form of the substances involved, and to the pH. At a constant pH (for all cases except where n and k = 1) a difference in potential of 0.03 volts (30°C) indicates a ten-fold increase (or decrease) in "hydrogen pressure". The term E'_o is frequently used. This is the E at a given pH (which should always be stated) when the oxidized and reduced materials are equal in quantity (50% oxidation or reduction), thus since:

$$E'_o = E_o + 2.3 \frac{RT\ (n-k)}{nF} \log H^+$$

or equation (58) becomes

$$(59)\quad E = E'_o + 2.3 \frac{RT}{nF} \log \frac{(Ox)}{(Red)}$$

The value of such measurements has been outlined by Axelrod and Johnson (1939), Barron (1939) and Clark (1934, 1938). In addition some knowledge of oxidation-reduction potentials are necessary in the cultivation of many bacteria (Allyn and Baldwin, 1932; Wood, Wood and Baldwin, 1935; Kligler and Guggenheim, 1938; Johnstone, 1940; Knight and Fildes, 1930; Vennesland and Hanke, 1940, and others).

Two methods of measurement are used. The first employs indicators, much in the manner of pH indicators. The color of the indicator serves to estimate the potential. These methods have been described in detail by Hewitt (1933) who lists suitable indicators. A similar list is given by Clark (1934, 1939). A selection of these is given in Table XXII. However, because of poising effects, relatively low color and catalytic and toxic effects of the dyes employed, the indicator system for measurement of oxidation-reduction potentials is somewhat less accurate than the comparable indicator method for the measurement of pH.

TABLE XXII

Representative Oxidation - Reduction Indicators

Indicator*	E'_o at pH 7
o - Chlorophenol Indophenol	+ 0.233
o - Cresol Indophenol	+ 0.191
1 - Naphthol - 2 - Sulfonate Indophenol	+ 0.123
Thionine	+ 0.062
Methylene blue	+ 0.011
Indigo tetrasulfonate	- 0.046
Indigo trisulfonate	- 0.081
Indigo disulfonate	- 0.125
Brilliant Alizarine blue	- 0.173
Phenosafranine	- 0.252
Safranine T	- 0.289
Neutral Red	- 0.325

*In virtually all cases the range from 98% oxidation to 98% reduction of the indicator is 0.1 volt.

The second method for the determination of oxidation-reduction potentials is the direct potentiometric measurement. For this a potentiometer is necessary and in most cases a vacuum tube potentrometer is essential. Suitable potentiometers are described by Allyn and Baldwin (1932) and Clark (1928) but most glass-electrode pH meters, being vacuum tube potentiometers, can be employed. In some, attachments are available for direct measurements; in others a calibration of the potentiometer in terms of millivolts must be undertaken. In the following discussion we will assume that a suitable potentiometer is available.

Electrodes for Oxidation-Reduction Potential Measurements: Suitable electrodes may be made by the fusion of approximately 1.5 inches of 23 gauge platinum wire to 12 inches of 24 gauge copper wire. Ends of the platinum and copper wires are held in a small flame from a blast lamp until the copper wire melts. At that moment the two wires are removed from the flame and immediately stuck together, end to end, and held in that position until the copper wire solidifies around the end of the platinum wire. It is best to handle the platinum wire with forceps instead of with one's fingers since it appears that something from the fingers causes a variation in potential readings from one electrode to another and this cannot be changed by any cleaning method tried.

The copper-platinum wire is inserted into a nine inch length of five mm.(o.d.) soft glass tubing in such a manner that a little more than one inch of platinum wire extends beyond the end of the tube. The end of the tube bearing the platinum wire then is sealed in a gas flame with the precaution that as thin a seal as possible is made to prevent subsequent cracking. While the glass is still soft, the platinum wire is pulled, drawing the glass seal to a short point thereby decreasing the thickness of the seal and permitting an imperfect seal to be readily detected. The platinum wire is twisted into a coil to provide as great a surface area as possible at a given level in the solution.

The electrodes may be cleaned in the usual chromic acid cleaning solution but it has been found that rinsing them in a solvent such as toluene or benzene followed by chromic acid solution followed by a dilute solution of hydrochloric acid results in the most consistent values. The electrodes are rinsed in distilled water after treatment in each of the solutions.

To determine whether the electrodes are suitable for use in a poorly poised solution, a number of electrodes are held at the same level in a vessel containing a solution of, for example, one per cent peptone. If potential determinations are taken at one-half hour intervals for two to three hours it will be found that the majority of the electrodes give a reading near one particular value. The electrodes that check within ten millivolts of each other may be used for future work.

Container for Test Solution: It has been found that a suitable container of convenient size can be prepared by blowing out a bulb 40 mm. in diameter at the base of a 25 x 200 mm. test tube. When the electrodes and salt bridge are placed in the tube so that they extend down into the bulb, the electrodes are not in contact with the walls of the container so that the container cannot affect the potential in the electrodes.

Fifty ml. of solution fills a tube of this type to a height 1 to 1.5 inches above the top of the bulb so that oxygen diffusion down to the level of the solution in the neighborhood of the electrode is negligible. In a system affected by atmospheric oxidation, potential measurements may be made over an extended period of time before the influence of oxygen is noticed. The effect of atmospheric oxidation may be excluded further by layering the solution with melted vaseline.

Salt bridge: The salt bridge may consist merely of an inverted J-tube with one arm long enough to reach the test solution and the other long enough to reach a vessel containing a solution of saturated KCl. It may be filled with saturated KCl-agar or may contain only saturated KCl if a piece of asbestos fiber is placed in the long end and the tube melted down around it to seal off the end. The fiber extending through the seal permits electron flow but prevents the KCl from escaping.

The salt bridge is recognized as a source of error in oxidation-reduction potential measurements, but the use of saturated KCl reduces the junction potential since the ionic velocities of K^+ and Cl^- are nearly equal and the saturated solution dominates the ionic migration at the interface.

Calomel half-cell: For the preparation of a calomel half-cell it is necessary that the HgCl, KCl and Hg are all of C. P. quality. About 100 ml. of saturated KCl is prepared with conductivity water. About one ml. of Hg and two gms of calomel are placed in a mortar and about 10 ml. of the KCl solution are added. The mixture is rubbed with a pestle, then allowed to stand until the calomel settles, the supernatant is decanted off and the procedure repeated five or six times. A tube of any size may serve as a container for the half-cell. If glass tubing is used, the platinum electrode may be sealed in the bottom and the potentiometer connected to the protruding end of the wire. A platinum electrode as described above will serve if a larger vessel such as a test tube is used. Mercury is placed in the vessel to a depth to cover the platinum and the Hg-calomel-KCl paste is transferred to the vessel which then is filled with saturated KCl. The salt bridge is attached and the half-cell sealed. Several types of calomel cells are shown in Fig. 42.

Sterilization of Electrodes: If potential measurements are to be made in a biological solution for a period of time it is necessary to use aspetic techniques. The electrodes are sterilized best by autoclaving them in distilled water with the electrodes and salt bridge mounted in either a rubber stopper or a firm cotton plug so that they may be transferred easily to the sterile test solution. If KCl - agar is used in the bridge, the long end of the tube may be placed in a small diameter test tube containing the KCl-agar and when the agar and glass tubing have cooled sufficiently following autoclaving the melted agar is drawn up into the bridge.

Measurement of potential: The test solution constitutes one half of a cell connected internally to the other half of the cell by the salt bridge. For standard reference the other half of the cell is a hydrogen half-cell, but the calomel half-cell, which gives a potential of +0.246 volts at 25°C. when referred to the hydrogen half-cell, is used for

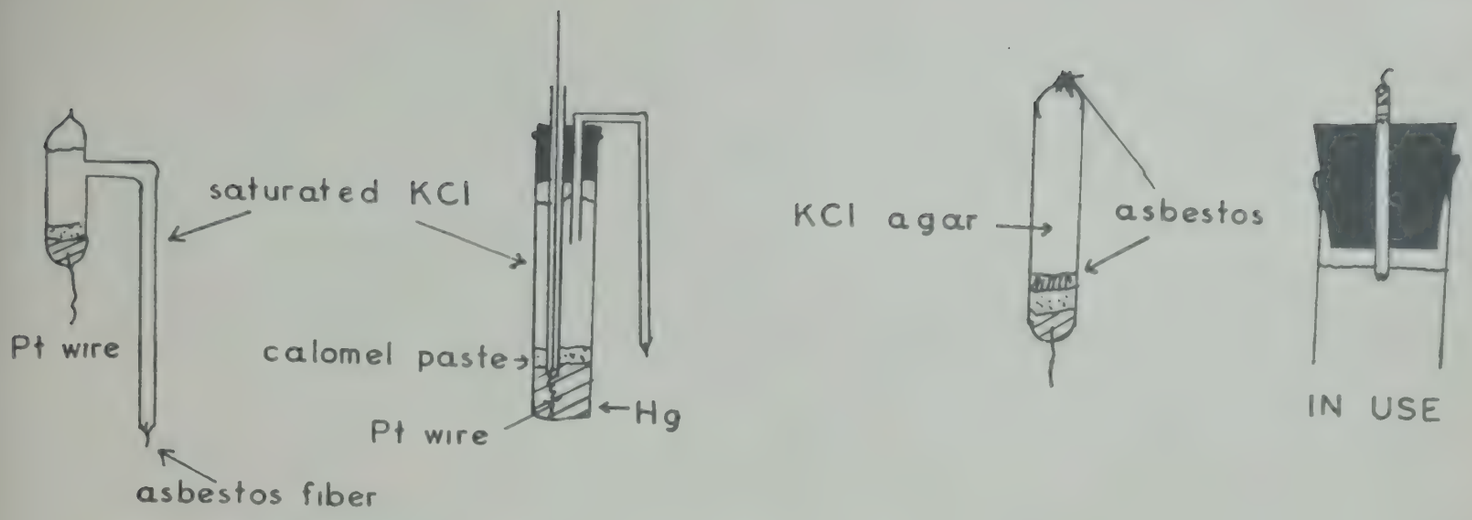

Fig. 42. Various Types of Calomel Cells (see text for description).

convenience. Oxidation-reduction values, however, should be referred back to the hydrogen half-cell.

The platinum electrodes in the two half-cells, the reference half-cell and the unknown half-cell, are connected to a potentiometer for potential determination. For systems of high capacity where polarization of the electrodes due to current flow is of little importance any type of potentiometer may be used. However, most biological systems are poorly poised so that slight polarization of the electrode affects the potential of the system. In this case it is necessary to use a suitable null-point instrument such as a quadrant potentiometer or a vacuum tube potentiometer. The system, then, with a calomel half-cell, is:

Pt	electrode	Unknown solution	salt bridge	KCl solution	salt bridge	calomel half-cell	Pt electrode

Potentiometer

It is advisable to use two electrodes in each sample of the solution and at least two samples of a given solution for the determination of its potential.

Details of measurement of oxidation-reduction potentials are given by Hewitt (1933) and Clark, et.al., (1928). Zerfas and Dixon (1940) have described a cell for such measurements and the results obtained are described by Dixon and Zerfas (1940). Hanke and Katz (1943) have described an electrolytic method for controlling the oxidation-reduction potential.

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Chapter Fourteen

MANOMETRIC ESTIMATION OF METABOLITES AND ENZYME SYSTEMS

P. P. Cohen

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Chapter Fourteen

MANOMETRIC ESTIMATION OF METABOLITES AND ENZYME SYSTEMS

INTRODUCTION

The popular usage of manometric equipment for measuring the overall metabolic gas exchange of biological systems has overshadowed to a considerable extent the great usefulness of this equipment for more specific chemical determinations. As has been previously pointed out, any reaction which results in either the production or utilization of a gas can be followed with great accuracy using a manometric apparatus. Further, reactions which give rise to end products having acidic groups can be accurately followed by allowing the reaction to take place in a bicarbonate medium.

In most manometric experiments involving biological transformations the quantity of metabolite involved is usually of the order of 0.5 to 5 mg. The manometric equipment is particularly suitable for semi-micro determinations in this range.

The recent development of micro-colorimetric methods and equipment has tended to replace older manometric methods. In many cases this represents a welcome improvement. However, there still remain a large number of metabolites which are peculiarly suited for manometric estimation. This is particularly true where specific enzyme systems can be utilized. Thus, the important metabolite succinic acid can be accurately determined in small quantities only by means of a manometric method employing a succinoxidase preparation. In certain instances one may have a choice of an equally suitable colorimetric or manometric method. For example, urea can be readily determined colorimetrically (Barker, 1944) or manometrically with urease at pH 5 (Krebs and Henseleit, 1932). The choice of one or the other method will be determined by the type of equipment available, the experimental set-up, etc. In the author's experience with studies of urea synthesis it was found to be more convenient to use the manometric technique, since the estimation could be carried out directly in the same vessels after removal of the tissue. Obviously one's decision to use one or the other analytical method will be determined to a considerable extent by the experience of the investigator, the type and amount of equipment available, the accuracy requirements of the experiment, etc.

SUCCINIC ACID

Principle: This method was first worked out by Szent-Gyorgyi and Gozsy (1935) and simplified and improved by Krebs (1937). In principle the method depends upon the extraction of succinic acid by means of ethyl ether. The succinic acid is then oxidized by means of a succinoxidase preparation and the O_2 consumption measured. The following reaction takes place (60):

$$(60)\quad COOH\text{-}CH_2\text{-}CH_2\text{-}COOH + 1/2\ O_2 \longrightarrow COOH\text{-}CH = CH\text{-}COOH + H_2O$$

The specificity of the method is insured by (1) washing the succinoxidase preparation free of coenzymes, and (2) the insolubility in ethyl ether of coenzymes present in the biological system.

Preparation of the sample for extraction: It is desirable to add a deproteinizing agent to the sample before extraction in order to get away from emulsification at the ether-water interface. This is best accomplished with sulfuric acid and sodium tungstate, as used in standard methods for blood analysis. Where the succinic content is low it is best to deliver the total sample into the extraction apparatus and then add the deproteinizing reagents and extract directly. To insure an adequate acidity, it is necessary to add an excess of H_2SO_4, usually 1-2 ml. of a 10% solution.

Extraction with ethyl ether: It is essential that the ethyl ether be freed of peroxides before using. This is best accomplished by storing a large quantity of ether over

metallic sodium and freshly distilling portions as needed.[1] This involves little trouble if a condenser and a distillation and receiving flask are permanently fixed at the steam bath used for the extraction apparatus. Such a set-up allows for efficient recovery of the ether remaining after the completion of the extraction. The removal of peroxides is essential since the succinoxidase preparation has a potent catalase activity, and the liberation of oxygen by this system will interfere in the manometric estimation of oxygen consumption.

A simple and efficient extraction apparatus is that of Kutscher and Steudel as illustrated in Fig. 43. The extractors may be made to contain different volumes of from 15 to 50 ml. The latter size has been found convenient and adaptable for extracting volumes of from 10-35 ml. Since the efficiency of extraction is determined in part by the height of the aqueous column, funnels of different diameters can be employed. Thus with small aqueous volumes, a funnel of wide diameter when filled with ether will heighten the aqueous column outside the funnel very considerably and so enhance the extraction efficiency. The efficiency of extraction is determined by such other factors as the rate of boiling, the temperature, the fineness and speed of the ether bubbles passing through the aqueous layer, etc. It is essential that the efficiency of extraction be determined for each extractor by estimating the recovery of known amounts of succinic acid. As an aid in estimating the rate of extraction, indicators with suitable partition coefficients may be employed (see Krebs, Smyth and Evans, 1940). The author has found that the addition of a few drops of phenol red (0.01% aqueous) to the fluid to be extracted will serve not only as an extraction indicator (about 60 per cent of the succinic acid will have been extracted when the phenol red disappears from the aqueous phase) but in addition serves as a neutralization indicator in the extract. It should be pointed out that at a low pH phenol red has a pink color not unlike that seen in the region of pH 7.

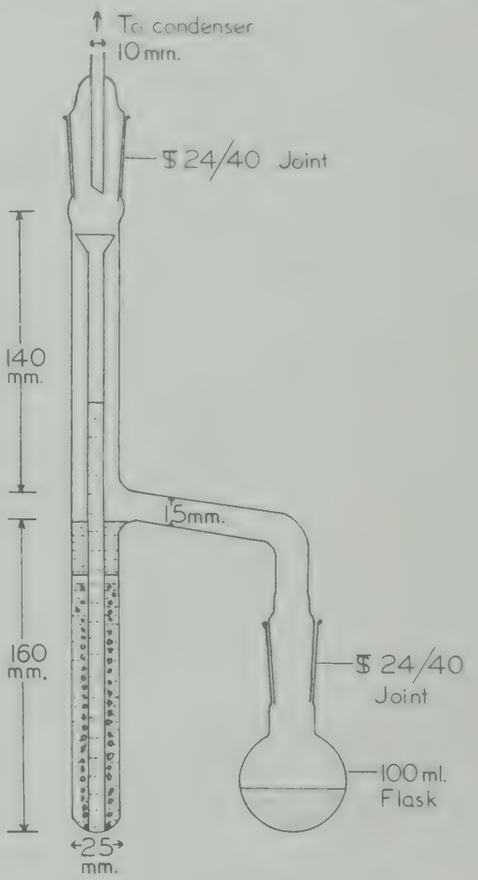

Fig. 43. Kutscher - Steudel Extraction apparatus.

When the extraction is completed, 1-2 ml. of 0.1 M phosphate buffer, pH 7.4 is added to the ether solution and the ether distilled off. The last traces of ether are removed by concentrating the aqueous residue on the steam bath to approximately 0.5 to 1 ml. The residue is then transferred to a small graduated cylinder (a graduated 15 ml. centrifuge tube is convenient) by means of a 1 ml. pipette. The flask is rinsed several times with small volumes (0.2-0.5 ml.) of 0.1 M phosphate buffer and the washings added to the contents of the graduated tube. Since the solution in the flask is colored due to the presence of the phenol red, the disappearance of the color in the successive washings can serve as a guide in determining the completion of transfer. The solution in the tube is now adjusted to the proper pH, i.e., 7.4, by the dropwise addition of dilute NaOH, if necessary. The final volume of the extract should be adjusted according to the succinic acid concentration. Since 1 ml. aliquots are usually employed, it is desirable that this amount should contain between 0.2 and 1 mg. of succinic acid. This would represent an uptake of 19 and 95 µl. of O_2 respectively, which is a convenient range.

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[1]The storage flask of ether plus sodium can be conveniently closed off by means of a "Bunsen valve". This is merely a short piece of rubber tubing sealed at one end with a tight fitting glass rod. The rubber tubing is slit with a razor blade, and then fitted over the glass tubing projecting from the cork stopper of the flask. When the pressure inside the flask increases it will permit escape of the gases (hydrogen or ether vapor) through the slit in the tubing.

Preparation of succinoxidase: A simple and suitable succinoxidase preparation can be made as follows: Pigeon breast, pig or sheep heart muscle is freed of fat and connective tissue. It is then coarsely ground in a meat chopper and suspended in 10 volumes of ice-cold distilled water. The suspension is frequently stirred during the first 10 minutes and then allowed to settle in the cold room. The supernatant is decanted and the residue sucked through a double layer of cheese cloth on a Buchner funnel. The muscle pulp is then resuspended in cold distilled water and the procedure is twice repeated. After the third washing, the muscle pulp is dried as completely as possible by suction. For use, a portion of the pulp is suspended in four to five times its weight of 0.1 M phosphate buffer (pH 7.4). This is best accomplished by grinding with a glass mortar and pestle. For storage, the muscle pulp is placed in a tightly covered container and allowed to freeze solid in the freezing compartment of a refrigerator. In this state the preparation remains active and with a low blank O_2 uptake for several weeks (Cohen, 1940a). In this connection it should be pointed out that freshly dissected pigeon breast or freshly killed mammalian heart muscle can be stored for months if frozen solid in covered containers and still yield very active succinoxidase preparations. Where fresh tissue is not readily available at all times this procedure is recommended to insure a continuous supply of enzyme. The phosphate suspension of the muscle pulp develops an appreciable blank O_2 uptake after 24 hours. Since the suspension is rather pasty, it is necessary to pipette it with a wide mouthed pipette. This is best accomplished by breaking off the fine tip from a 3 ml. pipette, flaming it, and then recalibrating to deliver 3 ml.

A dry preparation of succinic dehydrogenase has been described by Weil-Malherbe (1937). Brilliant cresyl blue is used as a carrier.

Manometric estimation of succinic acid: Warburg flasks of about 20 ml. capacity with a center well and a sidearm of 1 ml. capacity are usually employed. To the center well is added 0.2 ml. of 10% KOH plus a square of filter paper. The succinic acid solution, usually 1 ml., is added to the sidearm. The succinoxidase suspension, usually 3 ml. is pipetted into the main compartment. The control vessel is made up in the same way excepting that 1 ml. of 0.1 M phosphate buffer, pH 7.4 with a drop of phenol red, is placed in the sidearm. The bath temperature is usually 40°C. After a 10 minute shaking period with the stopcocks open for equilibration, the manometer fluid is adjusted so as to provide a maximum scale for reading and the stopcocks are then closed. Readings are then taken every 5 minutes until the O_2 uptake is constant in the different manometers. This may require anywhere from 1 to 5 successive readings depending on the temperature of the solutions, the rate of shaking, the thickness of the succinoxidase suspensions, etc. After equilibration is attained, the content of the sidearm is delivered into the main compartment and the manometers swirled once or twice to insure mixing. The manometers are then tipped back and forth once or twice to insure mixing of the solution remaining in the sidearm with the enzyme suspension. A small amount of the suspension is left in the sidearm to insure oxidation of the last traces of the succinic acid. Readings are then taken every 10 minutes until the Δ values (uptake per unit time) of the control and the experimental manometers are equal on two successive readings. The reaction is usually complete in 40 minutes. However, in the presence of high salt concentrations, high concentrations of fumaric, α-ketoglutaric and oxalacetic acids, the reaction rate is slowed up so that periods as long as 90-120 minutes may be required to complete the reaction.

Determination of succinic acid in the presence of malonic acid: It is usually necessary to add malonic acid to aerobic biological systems in which succinic acid synthesis is to be determined. This substance inhibits the oxidation of succinic acid. It is, therefore necessary to remove the malonic acid before the succinic acid determination can be carried out. This is most simply done by oxidation with acid permanganate using a procedure similar to that employed for α-ketoglutaric acid determination (see page 145). Malonic acid is readily oxidized by acid permanganate while succinic acid is not. If α-ketoglutaric acid is present in this system, which will be likely, it will be converted to succinic acid by the permanganate treatment and therefore will be included in the succinic acid determination. Should it be desired to determine succinic acid only, it is possible to remove the succinic and malonic acids from the α-ketoglutaric acid by the addition of $NaHSO_3$ in slight excess, and enough H_3PO_4 to make the solution 0.04 N. Since the sulfite addition product of α-ketoglutaric acid is relatively insoluble in ether, the succinic and malonic acids can be extracted with ether (Weil-Malherbe, 1937). After removal of the ether the malonic acid can be destroyed by oxidation with acid permanganate, leaving succinic acid (Krebs and Eggleston, 1940).

Analytical range: The smallest quantity of succinic acid which can be determined by the manometric method is limited chiefly by the accuracy of the manometric equipment. Since 0.05 mg. of succinic acid is equivalent to 4.75 µl. of O_2 uptake, this amount can be considered the lower limit of the method.

Calculation:

µl. O_2 uptake is converted to mg. of succinic acid as follows

$$\frac{\text{µl. } O_2}{112} \times 1.18 = \text{mg. succinic acid}$$

It should be noted that 1 µl. O_2 uptake is equivalent to 2 µl. succinic acid.

α-KETOGLUTARIC ACID

Principle: This method depends on the conversion of α-ketoglutaric acid, either as such or in the form of its dinitrophenylhydrazone, to succinic acid by oxidation with acid permanganate (Krebs, 1938) (equation 61). The succinic acid formed is then determined by means of a succinoxidase preparation as described in the previous section.

(61) $COOH\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}COOH \longrightarrow COOH\text{-}CH_2\text{-}CH_2\text{-}COOH + CO_2$

Reagents: 1) 50% H_2SO_4.

2) 10% Na_2WO_4.

3) 0.8 N H_2SO_4.

4) 3% $KMnO_4$

5) 2:4 dinitrophenylhydrazine, 1% dissolved in 10% H_2SO_4.

6) Reagents for succinic acid determination.

Procedure: If indicated, the α-ketoglutaric acid can be converted to its dinitrophenylhydrazone and extracted with ether as outlined by Krebs (1938). This is usually unnecessary with most biological systems since the concentration of metabolites which might interfere is not significantly great. In most experiments, therefore, one may proceed as follows. An aliquot of the deproteinized solution is delivered directly into the Kutscher-Steudel extractor, followed by 1 ml. of 50% H_2SO_4 plus 2 ml. of 3% $KMnO_4$. The solution is allowed to stand at room temperature for 30 minutes. If during this time the permanganate is decolorized, more is added. The solution is then directly extracted with ether. The ether extract usually contains considerable quantities of MnO_2. This, however, does not interfere with the succinic acid determination but, if desirable, can be readily removed by centrifugation after the ether is removed and the solution made up to volume. The procedure otherwise is the same as that outlined for succinic acid.

A succinic acid blank is determined by ether extraction of the protein free filtrate without permanganate treatment. When high concentrations of substrates, which react with permanganate to yield succinic acid are employed, it is necessary to run either a "zero time" blank with this substrate present, or to separate the α-ketoglutaric acid as its dinitrophenylhydrazone.

For separation of α-ketoglutaric acid as its dinitropheylhydrazone, the following procedure is employed. To an aliquot of the protein-free filtrate is added 1-2 ml. of 1% dinitrophenylhydrazine solution in 10% H_2SO_4. The solution is allowed to stand for 30 minutes and then extracted twice with 1/5 volume of ether in a separatory funnel. The ethereal solutions are combined and the ether removed by evaporation on the steam bath. The residue is dissolved in 2-5 ml. of 2N NaOH and transferred quantitatively to a graduated cylinder of 25-50 ml. capacity. The solution (usually about 20 ml.) is acidified with 50% H_2SO_4 to bring the acidity to about 1 N. 3 ml. of 3% $KMnO_4$ are then added and the solution allowed to stand at room temperature for about 30 minutes. If decolorization of the permanganate occurs during this interval, more permanganate solution (or solid

if the volume is to be kept within certain limits) is added. The final volume is then determined, the solution filtered and the succinic acid extracted with ether in the usual manner from an aliquot of the filtrate.

Specificity: Substances which yield appreciable amounts of succinic acid when oxidized with acid permanganate are α-hydroxyglutaric acid, arginine, butyric acid and glutamic acid. However, under most experimental conditions the concentration of these substances is never high enough to yield significant amounts of succinic acid.

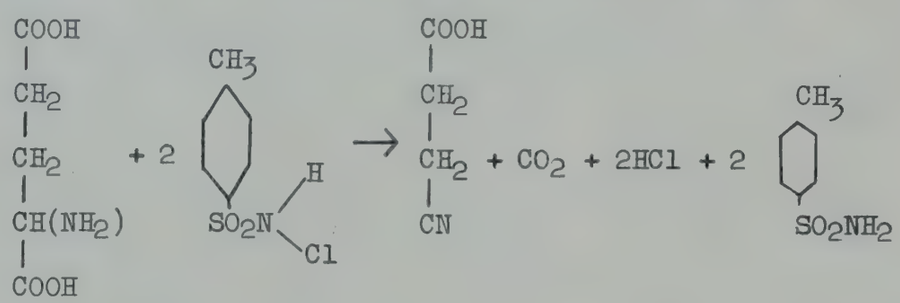
Calculation: The µl. O_2 uptake is converted to mg. of α-ketoglutaric acid as follows:

$$\frac{\mu l.\ O_2}{112} \times 1.46 = \text{mg. of } \alpha\text{-ketoglutaric acid}$$

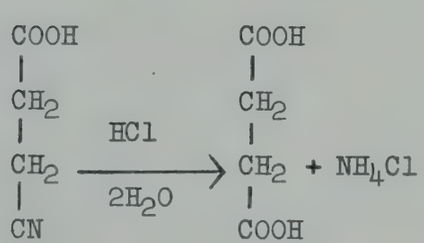
GLUTAMIC ACID

Principle: This method depends upon the conversion of glutamic acid by means of an excess of chloramine-T to β-cyanopropionic acid, and the hydrolysis of the latter to succinic acid according to equations (62) and (63) (Cohen, 1939). The succinic acid is then determined by means of a succinoxidase preparation as previously described.

(62)



(63)



Reagents:

1) Citrate buffer, pH 4.7. (17.65 grams $Na_3C_6H_5O_7 \cdot 2H_2O$ and 8.40 grams of $C_6H_8O_7 \cdot H_2O$ are dissolved in H_2O and diluted to 50 ml.
2) 0.1M phosphate buffer, pH 7.4. (17.8 grams $Na_2HPO_4 \cdot 2H_2O$ are dissolved in about 500 ml. of H_2O and 20 ml. of 1 N HCl added. The solution is then diluted to 1 L.)
3) 10% chloramine-T (N-chloro-p-toluenesulfonamide) must be freshly prepared before use.
4) conc. HCl.
5) 5% NH_4Cl solution.
6) Sat. NaOH solution (50%).
7) Reagents for succinic acid determination.

Procedure:

1) Deproteinization
When tissue slices are employed it is not necessary to deproteinize since the small amount of protein present does not interfere. Tissue minces, homogenates, etc. are deproteinized with H_2SO_4--Na_2WO_4 solutions. An aliquot of the filtrate is used for the determination.
2) Oxidation by chloramine-T
The solutions to be analyzed are brought to pH 4.7 by the addition of 1-1.5 ml. of citrate buffer. 2 ml. of freshly prepared 10% chloramine-T are added and the solutions well mixed by shaking. They are then placed in a rack and

shaken at 40° for 10 minutes. The reaction is conveniently carried out in small Erlenmeyer flasks, or, where tissue slices are employed, the reaction may be carried out in the manometric flasks after removal of the slices and the alkali in the center well. After 10 minutes shaking the containers are removed and placed in an ice bath for 15-20 minutes to precipitate most of the p-toluene-sulfonamide formed as a reaction product, and most of the unused chloramine-T. The solutions are filtered while cold, the precipitate washed with several small volumes of cold water, and the combined filtrates and washings collected in large test tubes (25 x 200 mm.).

3) Hydrolysis of β-cyanopropionic acid

Conc. HCl is added to the filtrate to make a final concentration of not less than 12.5%. The tubes are covered and placed in a boiling water bath for 15 minutes after which time they are removed and allowed to cool. Conc. NaOH is added dropwise until the solution becomes hot. At this point 0.5 ml. of 5% NH_4Cl solution is added and the contents well mixed. The NH_4Cl decomposes traces of chloramine-T which if present will decolorize the indicator. The solution is cooled and a few drops of phenol red are added. The solution is then made alkaline to a purple color. A large excess of alkali should be avoided as the p-toluene-sulfonamide forms a salt in strongly alkaline solution. The solution is then transferred to a Kutscher-Steudel extractor and extracted with ether. The alkaline solution is extracted with freshly distilled ether for a time sufficient to remove the remaining traces of p-toluene-sulfonamide (usually 1-2 hours). After this time, the extraction flasks are removed and replaced by clean ones. The contents of the extractors are then acidified with 2-3 ml. of 10% H_2SO_4. The phenol red will change to a light yellow-pink color. Additional ether is then added and the extraction resumed for two hours or more. (See discussion under succinic acid determination).

Preparation of solution for succinic acid determination: Essentially the same procedure is followed as that outlined under succinic acid determination.

Calculation: The µl. O_2 uptake is converted to mg. of glutamic acid as follows:

$$\frac{\mu l.\ O_2}{112} \times 1.47 = \text{mg. glutamic acid}$$

Specificity of the method: Aside from succinic, only glutamine and glutathione are known to interfere with the determination of glutamic acid. Succinic acid is readily taken care of by extraction of the solution without chloramine-T treatment. This will represent a succinic acid blank. Since glutamine will be encountered in appreciable quantities only under rather special conditions (Krebs, 1935a) this substance will present few difficulties. Glutathione, on the other hand, is present in fairly high concentration in most tissues. In experiments where glutamic acid formation or disappearance is being measured, a "zero time" blank will take care of the glutathione content of the tissue. However, if the absolute concentration of glutamic acid is desired, it is necessary to separate the β-cyanopropionic acid from the glutathione homologue by ether extraction. For details of this, consult the original paper (Cohen, 1939).

ASPARTIC ACID FORMATION AND DISAPPEARANCE IN TRANSAMINATION SYSTEMS

As can be seen from Reaction 62, glutamic acid yields 1 mole of CO_2 when it reacts with an excess of chloramine-T at an acid pH. This is true of most of the other amino acids with the exception of glycine and aspartic acid which yield 2 moles of CO_2 (Cohen, 1940b). Since transamination is concerned chiefly with the reaction, (64) it is possible to

(64)
$$\begin{matrix} COOH \\ CH_2 \\ CH_2 \\ CH(NH_2) \\ COOH \end{matrix} + \begin{matrix} COOH \\ CO \\ CH_2 \\ COOH \end{matrix} \rightleftharpoons \begin{matrix} COOH \\ CH_2 \\ CH_2 \\ CO \\ COOH \end{matrix} + \begin{matrix} COOH \\ CH(NH_2) \\ CH_2 \\ COOH \end{matrix}$$

determine aspartic acid formation and disappearance in transaminating systems with the above substrate combinations. For purpose of rapid assay of any tissue or cells for

transamination activity, or for following activity in purification procedures, the method is both accurate and rapid. For analytical details the reader is referred to the paper by Cohen, (1940b). This method has been used for study of transamination in animal tissues (Cohen and Hekhuis, 1941), in plant tissues (Albaum and Cohen, 1943) and in bacteria (Lichstein and Cohen, unpublished).

FUMARIC (AND MALIC) ACID

Principle: (Krebs, Smyth and Evans, 1940). Fumaric acid is reduced to succinic acid in the presence of zinc and phosphoric acid (65),

$$(65)\quad COOH\text{-}CH = CH\text{-}COOH \xrightarrow{H_2} COOH\text{-}CH_2\text{-}CH_2\text{-}COOH$$

The formed succinic acid is extracted with ether and determined manometrically by means of a succinoxidase preparation. The concentration of malic acid is calculated from the equilibrium constant of the system (65a).

$$(65a)\quad COOH\text{-}CH{=}CH\text{-}COOH \underset{}{\overset{H_2O}{\rightleftharpoons}} COOH\text{-}CHOH\text{-}CH_2\text{-}COOH$$

Reagents:

1) Meta-phosphoric acid, 5% solution (made up without heating; can be stored in ice box for 1-2 weeks).
2) Zinc filings, 20-30 mesh.
3) $CuSO_4 \cdot 5H_2O$, 20% solution.
4) 10 M phosphoric acid solution (100 ml. phosphoric acid, sp. gr. 1.75, plus 158 ml. H_2O).
5) Reagents for succinic acid determination.

Procedure: The tissue suspension, or similar material, is deproteinized at the temperature of the experiment in which the fumarate was formed. (This is necessary in order to insure no disturbance of the equilibrium of the system shown in Reaction 65a). Deproteinization is carried out by the addition of 1/5 volume of 5% metaphosphoric acid. An aliquot of the filtrate is transferred to a Kutscher-Steudel extractor, or a measuring cylinder, and 0.5 grams of zinc filings, 2.3 ml. of phosphoric acid and 0.25 ml. of CuSO4 solution are added per 10 ml. aliquot. (Excessive frothing can be controlled with a drop of capryl alcohol). After 60 minutes, when the greater part of the zinc has been decomposed, the formed succinic acid is extracted directly with ether. The succinic acid so extracted is then estimated as outlined previously.

Calculations: Since fumaric and malic acids exist in equilibrium in tissues containing fumarase it is possible to estimate the sum of these two by use of the equilibrium constant. The factors for this equilibrium at different temperatures are shown in table XXIII. In systems where fumarase is not present, such as purified biological systems, obviously it is not necessary to use these factors. The µl. of O_2 uptake is converted into mg. of fumaric acid as follows:

$$\frac{\mu l.\ O_2}{112} \times 1.16 = \text{mg. fumaric acid}$$

Specificity: Malic, tartaric, oxalacetic, aspartic, glutamic, citric and aconitic acids do not form succinic acid under the conditions of this method. Maleic acid behaves like fumaric acid, but since it is not present in biological material it need not be considered. If the solution contains succinic acid, it must be determined separately in an aliquot before the treatment with zinc and acid and deducted from the succinate found in the fumaric acid determination.

TABLE XXIII

Factor by which the fumaric acid (µl.) found in the presence of fumarase is to be multiplied in order to obtain the sum of fumaric and malic acids (µl.) present.

Temp. 0°C	Factor
50	3.65
40	4.17
30	4.54
20	5.57

PYRUVIC ACID

Principle: The manometric estimation of pyruvic acid is based on the production of CO_2 by enzymatic decarboxylation (Warburg, et al, 1930; Westerkamp, 1933) at an acid pH (reaction 66).

$$(66)\quad CH_3COCOOH \xrightarrow{\text{Carboxylase}} CH_3CHO + CO_2$$

The carboxylase method is particularly suitable for the rapid determination of pyruvate in certain biological systems.

Reagents:

1) Yeast extract.
Freshly pressed brewers yeast is spread on filter paper and dried at room temperature with the aid of a fan. If quickly dried and stored in the ice box, the dried yeast will yield active carboxylase preparations for as long as 3 years. For preparation of the extract, 10 grams of dried yeast are suspended in 30 ml. of distilled water by grinding in a mortar. The suspension is then poured into a tall cylinder (100 ml. graduate is suitable) and allowed to stand in a water bath at 30°C. for 1-2 hours, or until the endogenous fermentation has ceased. The suspension is then centrifuged at fairly high speeds for 15 - 20 minutes. The milky supernatant liquid is removed by decantation. It is treated with 1/20 volume of acetate buffer and freed of dissolved CO_2 by shaking the container while attached to a water vacuum pump. The carboxylase activity of such a preparation decreases rapidly on storage even at 0°C. Stabilization on storage at 0°C. for almost 1 week may be accomplished by adding 1/5 vol. of 90% glycerol. (Schoenebeck and Neuberg, 1935). In the author's experience it has been found more expedient to make up fresh preparations as needed.
2) 3M Acetate buffer, pH approximately 5.
2 parts of $NaC_2H_3O_2 \cdot H_2O$ plus 1 part glacial acetic acid.
1/10 volume of M/2 KH_2PO_4 is added to the buffer.

Procedure: If tissue breis, minces, homogenates, or bacterial suspensions are being used in studying pyruvic acid metabolism, the system is acidified with 1 ml. of acetic acid--acetate buffer, transferred to a graduated centrifuge tube and made up to a convenient volume with washings. Where pyruvic acid disappearance is being studied and a high concentration of pyruvic acid is present, it is necessary that the dilution be great enough so that the CO_2 production from the aliquot will not exceed the capacity of the manometer. Thus, if one were to add 1344 µl. of pyruvic acid (0.3 ml. of 0.2 M solution) to a system and then measure pyruvic acid disappearance, an aliquot of approximately 1/5 the total volume should be taken. This would represent, assuming no disappearance, a production of 269 µl. CO_2. If the expected disappearance is great, the final volume should be kept down accordingly so as to permit the use of a constant volume aliquot, usually 2 ml. In experiments with tissue slices it is only necessary to remove the slices and then add the acetic acid--acetate buffer and make up to volume. With very heavy tissue and bacterial suspensions it is desirable to centrifuge after adding buffer and making up to volume. An aliquot (usually 2 ml.) of the supernatant is then employed.

The manometric flasks are usually set up to contain a 2 ml. aliquot of the acidified incubation system in the main compartment. The side arm contains 0.5 ml. of the yeast extract. Bath temperature is usually set at 25°C. The control vessel contains 2 ml. of water treated with 1/10 volume of acetic acid--acetate buffer in the water compartment in place of the incubation system.

It is necessary to equilibrate until all CO_2 production ceases, or is constant in both the control and experimental vessels. Readings are taken every 5 minutes after the taps are closed. The solutions are then mixed, and readings again taken every 5 minutes. The reaction is usually complete in 10-15 minutes. As a matter of fact, significant CO_2 production beyond this time usually means that the carboxylase system is weak, or that other substrates, such as ketoglutaric or oxalacetic acids are reacting.

Calculations: µl. of CO_2 produced is converted into mg. of pyruvic acid as follows:

$$\frac{\text{µl. } CO_2}{224} \times .88 = \text{mg. of pyruvic acid.}$$

Specificity: Most of the α-ketonic acids will yield CO_2 when added to yeast extract. However, the rates of reaction vary considerably, pyruvic acid reacting at the fastest rate. A comparison of the relative rates for different α-keto-acids can be seen from the following table (XXIV):

TABLE XXIV

Decarboxylation of α-keto acids

Keto acid	Per cent theoretical CO_2 production in 15 minutes
Pyruvic	100
α-ketobutyric	90
α-ketoglutaric	8
oxalacetic	20
acetopyruvic	60

It is apparent from the preceding table that the determination of pyruvic acid in systems containing relatively large amounts of other α-keto acids will present some difficulty. Since α-ketobutyric acid will not normally be present in measurable amounts, this compound will not be a source of error. However, if one attempts to measure pyruvate in a system where relatively large quantities of oxalacetic and acetopyruvic acids are present, it may be difficult to obtain accurate pyruvate values by the carboxylase method. On the other hand, this technique will be found to be particularly valuable in experiments in which the chief reaction product is pyruvic acid. As examples of the former the experiments of Krebs and coworkers (1937-1940) on pyruvic acid metabolism in different tissues, and those of Cohen (1940a,b) on transamination may be cited. A production of pyruvic acid from lactic acid by means of lactic dehydrogenase preparations would be an example of the latter type.

OXALACETIC ACID

Principle: This method is based on the fact that β-ketonic acids are catalytically decomposed by primary amines in an acid medium to yield CO_2 (reaction 66a).

$$(66a)\quad RCOCH_2COOH \longrightarrow RCOCH_3 + CO_2$$

Ostern (1933) first applied this principle to the determination of oxalacetic acid using aniline as a catalyst. The reaction was carried out in acetic acid--acetate buffer, pH 5, and at 5°C. Quastel and Wheatly (1933) introduced the use of aniline hydrochloride for the determination of aceto-acetic acid. Edson (1935) employed aniline citrate for the same reaction, to better advantage. The use of aniline citrate for oxalacetic acid determination was reported by Greville (1939). The advantage of aniline citrate over that of other aniline salts is the greater solubility of this compound which insures a high concentration of the catalyst.

It should be borne in mind that oxalacetic acid has an appreciable rate of spontaneous decarboxylation. The influence of pH, various salts, and temperature on this reaction have recently been reported by Krebs (1942a).

Reagents:

1) 50% citric acid solution.
2) Aniline citrate: equal volumes of 50% citric acid and aniline (make up at time of using).

Procedure: In the presence of thick tissue suspensions, deproteinization is conveniently accomplished with 10% trichloracetic acid. In experiments with thin suspensions, deproteinization is not essential, the reaction being stopped by the addition of 0.3 ml. of 50% citric acid. A similar procedure is employed where tissue slices are used, the slices being removed and 50% citric acid added to the flask contents directly. It is essential that at the end of the experimental incubation period the flasks be placed in an ice bath and that all manipulations up to the point of the manometric oxalacetic acid determination be carried out at the lowest possible temperature.

In experiments designed to measure oxalacetic production from added metabolites, it will usually be possible to measure the total oxalacetic acid content of the incubation system. In such instances the measurment is carried out in the same manometric flask. In systems which will be expected to yield oxalacetic acid in amounts exceeding the capacity of the manometers, an aliquot must be employed.

The main compartment of the manometric flask contains 2-3 ml. of the solution to be tested (either an aliquot of the trichloracetic acid filtrate, or the incubation mixture itself) plus 0.3 ml. of 50% citric acid. If alkali has been used in the center well during the incubation, this must be removed by wiping out with filter paper. It is advisable in such cases to add 0.2 ml. of 50% citric to the center well to insure the complete absence of free alkali. The sidearm contains 0.4 ml. of the aniline citrate solution. A control vessel contains distilled water in place of the incubation mixture.

If equipment for maintaining the bath at 5°C. is available, it is preferable to carry out the determination at this temperature. At this temperature, the reaction is quite specific. At higher temperatures other β-ketonic acids (such as aceto-acetic and acetone dicarboxylic acids) react. In experiments where these compounds can be expected to be present in considerable quantities, the use of low temperatures is essential. However, in most instances this will not be the case, and consequently one may choose the lowest bath temperatures which the equipment and environment will permit. Ordinarily temperatures of 20-22°C. can be maintained without difficulty.

An initial equilibration period of 5 minutes with the taps open is necessary to insure removal of dissolved CO_2. After the taps are closed, readings are taken every 5 minutes until constancy is reached. The flask contents are then mixed. Readings are then taken as usual until no more CO_2 is produced, or until the pressure changes in the control and experimental flasks are constant. Immediately on mixing a negative pressure will usually result due to the difference in density of the aniline citrate solution and the solution in the main compartment. This will be taken care of by the control vessel, which contains aniline citrate plus water.

Calculations: Microliters of CO_2 produced is converted into mg. of oxalacetic acid as follows:

$$\frac{\mu l.\ CO_2}{224} \times 1.32 = \text{mg. of oxalacetic acid.}$$

Specificity: As mentioned above, aniline salts catalyze the decomposition of most β-ketonic acids. In most biological systems, aceto-acetic acid will be the chief interfering compound. However, the relative rate of oxalacetic acid decarboxylation is so much more rapid than that of aceto-acetic acid that at low temperatures the reaction is practically specific for oxalacetic acid.

ACETOACETIC ACID

This determination is identical with that for oxalacetic acid, excepting that the reaction is carried out at higher temperatures. (35-40°C.). The technical details as outlined by Edson (1935) are suitable for most experiments.

XANTHINE AND HYPOXANTHINE

Principle: (Krebs and Orstrom, 1939). Xanthine and hypoxanthine are oxidized to uric acid by means of a purified xanthine oxidase preparation. O_2 uptake and uric acid formation are measured. From the ratio of O_2 uptake to uric acid formation the amounts of xanthine and hypoxanthine can be calculated. The former compound requires 2 atoms of oxygen, the latter, 1 atom for oxidation to uric acid.

Reagents:

1) Xanthine oxidase: This preparation must be highly active. A suitable preparation is made from fresh milk as follows (Dixon and Kodama, 1926): a liter of milk is clotted with renin and the clot broken up with a glass rod. The whey is filtered off through muslin. Solid ammonium sulfate is added to the amount of 215 grams for each 880 ml. of whey. After standing for 30-60 minutes the underlying clear solution is sucked off as completely as possible. The supernatant globulin layer is now centrifuged and the solid floating cake transferred to a filter paper and dried *in vacuo*. The dried material is freed of fat by ether extraction and the residue dried again. The enzyme keeps for several weeks if stored *in vacuo*. The yield is usually of the order of 3-5 grams per liter of milk. For use, the enzyme is made up as a 10% solution in 0.1 M phosphate buffer, pH 7.4. The activity of the preparation should be tested with a standard hypoxanthine solution. 15 mg. of the enzyme (0.15 ml. of 10% solution) should be capable of oxidizing 0.5 mg. of hypoxanthine in less than 1 hour (40°, pH 7.4).
2) Aqueous Pigeon liver extract: 1 gram of pigeon liver is thoroughly ground with sand and 10 parts of water and centrifuged. The supernatant liquid containing an active catalase, is used. The solution if preserved with octyl alcohol and stored in the refrigerator will remain active for several weeks. Mammalian liver is not suitable since it may contain uricase.
3) Phosphate buffer, 0.1 M, pH 7.4.
4) Reagents for uric acid determination: Uric acid may be determined by any one of several methods:
   a) conversion of uric acid to urea and the determination of the latter manometrically (Edson and Krebs, 1936).
   b) The Folin colorimetric method using uricase to insure specificity (Blauch and Koch, 1939).

Procedure: The sample to be analyzed is adjusted to pH 7.4. (This need be approximate only, since the xanthine oxidase activity does not vary significantly between pH 6.8 and 7.8). The main compartment of the manometric flasks contain 0.5 ml. of phosphate buffer, 0.5 ml. of liver extract, 3.5 ml. of the solution to be tested, and 0.15 ml. of xanthine oxidase solution. The center well contains 0.2 ml. of 10% KOH. As a blank, a flask is set up which contains all the constituents except that 3.5 ml. of water is used in place of the solution being tested. The bath temperature is 40°. The O_2 uptake should be complete in less than 60 min. and should agree with the theoretical value within 2%.

Calculations: On the basis of hypoxanthine requiring 1 mole and xanthine 0.5 mole O_2 for conversion to uric acid, the following equations may be set up:

$$X_{\text{hypoxanthine}} = 2X_{O_2} - X_{\text{uric acid}}$$

$$X_{\text{xanthine}} = 2\,(X_{\text{uric acid}} - X_{O_2})$$

(all subscripts refer to moles.)

Specificity: Of the many substances which may absorb O_2 in the presence of xanthine oxidase, only hypoxanthine, xanthine, adenine and a few aldehydes are known to occur in biological material. The presence of aldehydes is indicated if an absorption of O_2 but no formation of uric acid occurs. It is unlikely that, in animal tissues at least, aldehydes will be present in quantities sufficient to interfere. Adenine, if present, will be determined as hypoxanthine. However, its presence will be suggested by its slow rate of oxidation. Adenine is oxidized at 1/20 the rate of hypoxanthine.

Since tissue extracts may contain appreciable quantities of nucleotides, nucleosides, or guanine, all of which liberate hypoxanthine or xanthine, the bound purine bodies may represent a potential source of error. In the application of the method by Krebs and Orstrom to pigeon liver preparations, no difficulties on this basis were encountered.

In addition to substances which may react directly with xanthine oxidase preparations, a number of compounds may interfere with the quantitative determination of O_2 uptake due to a coupled oxidation. Alcohols, hemoglobin, methemoglobin, nitrites, etc. react in such a system due to the fact that they are readily oxidized by the hydrogen peroxide formed as an end product of the xanthine and hypoxanthine oxidation. Of these substances hemoglobin and its derivatives are the only ones likely to be present in animal tissues. This coupled oxidation is inhibited within certain limits by the use of liver extract which because of its high catalase activity rapidly decomposes the hydrogen peroxide as it is formed.

The lower limit of the analytical range of this method is 0.1 mg.

UREA

Principle: (Krebs and Henseleit, 1932). Urea is converted by urease to ammonium carbonate at pH 5. The ammonium carbonate in turn reacts to yield CO_2 according to the following equation (67).

(67) $$\begin{matrix} NH_2 \\ | \\ CO \\ | \\ NH_2 \end{matrix} \xrightarrow{\text{urease}} (NH_4)_2CO_3 \xrightarrow{2H^+} 2(NH_4)^+ + H_2O + CO_2$$

The CO_2 formed is measured manometrically.

Reagents:

1) Urease. While urease itself is highly specific in its action, the ordinary sources of urease may contain enzyme systems which will yield CO_2 under similar conditions. In particular, carboxylase may yield CO_2 from pyruvic acid under the above conditions. Unpublished experiments by the writer have shown that commercial jack bean meal contains a potent carboxylase system. Soy bean meal extracts, on the other hand, are practically devoid of this enzyme. The carboxylase activity of the jack bean meal extract is readily lost on dialysis against dihydrogen sodium phosphate solution without influencing the urease activity. In practice, however, it has been found far more convenient to purchase a purified urease (Arlco urease, made by Arlington Chemical Co., Arlington, Mass.) which is free of carboxylase. Since this material is stable for a long time and requires no preparation other than solution before use, it is more economical from the standpoint of both time and money. It is best prepared in a concentration of 10 mgs. per ml. of solution, the latter being 9.5 parts distilled water plus 0.5 parts acetic acid--sodium acetate buffer, pH 5.
2) Acetic Acid-Sodium Acetate Buffer: 27.2 g. sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) plus 6 g. glacial acetic acid are made up to 100 ml. with distilled water. This represents a 3N acetic-ion concentration and has a pH of 5.

Procedure: The tissue slices are removed from the flasks and the remaining solution acidified with 0.3 ml. of acetic acid-sodium acetate buffer; 0.5 ml. of urease solution is added to the sidearm. The control vessel contains distilled water plus acetic acid-sodium acetate buffer in place of the experimental solution. After a suitable equilibration period, the solutions are mixed and the manometric changes observed. The reaction

is usually run at 38° with air as a gas phase. The reaction should be completed in 20-30 minutes.

Calculation: The µl. CO_2 produced is converted to mg. urea as follows:

$$\frac{\mu l.\ CO_2}{224} \times 0.60 = \text{mg. urea.}$$

Specificity: As previously pointed out, in the presence of pyruvate and related substances, high "urea" values may be obtained because of carboxylase activity in the crude urease preparations. By the use of purified urease preparations this difficulty is eliminated. Krebs (1942b) has recently called attention to another source of CO_2 unrelated to urea, i.e., aceto-acetic acid. This substance is found in considerable amounts when liver slices are incubated in the presence of ammonium salts (Edson, 1935). Since aceto-acetic acid slowly decomposes to yield CO_2, it may interfere with the urea determination. In order to destroy the acetoacetic acid, Krebs recommends the addition of 0.1 ml. of aniline plus 1 ml. of acetic acid-sodium acetate buffer to the unknown solution. The aniline catalyzes the decomposition of the acetoacetic acid so that it is completely broken down within the usual equilibration period of 20 minutes. (See method for acetoacetic acid determination).

GLUTATHIONE

Principle: (Woodward, 1935). This method depends on the fact the glyoxalase system requires reduced glutathione as a coenzyme. The rate of conversion of methylglyoxal to lactic acid is dependent on the concentration of glutathione within certain limits. The reaction is carried out in a bicarbonate - CO_2 system. The CO_2 formed as a result of the conversion of methyl glyoxal to lactic acid is measured manometrically (reaction 68).

$$(68)\quad CH_3COCHO \xrightarrow[GSH]{H_2O} CH_3CHOHCOOH$$

Reagents:

1) Acetone dried yeast: This is prepared according to Albert, et al. (1902). 500 grams of starch-free bakers' yeast is coarsely pulverized and passed through a sieve (10 mesh) into 3 liters of acetone in a flat dish. After stirring, the yeast is allowed to remain in the acetone for 10 minutes. The acetone is then decanted and the residue sucked dry on a hard filter paper. The resulting cake is again broken up and suspended in one liter of acetone for 2 minutes. After decanting the supernatant the yeast residue is sucked dry. The resulting cake is then broken up and covered with 250 ml. of ethyl ether and mixed for 3 minutes after which the suspension is filtered by suction. The yeast cake is finely pulverized and spread out in a thin layer on filter paper for 1 hour. The resulting powder is then further dried by placing in a desiccator for 24 hours at 45°. Before use, the yeast must be freed of glutathione. This is most readily done by suspending 1 gram of dried yeast in 50 ml. of distilled water and centrifuging. The supernatant is decanted and the centrifugate suspended again with 50 ml. of distilled water. After centrifuging again and decanting, the yeast is made up as a 15-20 per cent suspension and stored in the ice box.
2) Methyl glyoxal: A solution of methyl glyoxal is readily prepared by distilling dihydroxyacetone from H_2SO_4 according to Neuberg, et al. (1917). 0.5 grams of dihydroxyacetone is introduced into a 100 ml. distilling flask. A mixture of 5 grams H_2O plus 1 gram of H_2SO_4 is placed in a small separatory funnel which is tightly fixed in the flask neck with a rubber stopper. The distilling flask is connected with a small efficient water-jacketed condenser. The H_2SO_4 solution is added dropwise while the flask is being heated. After 5 ml. of distillate has come over (the distillate is best collected in a glass stoppered graduated cylinder) 5 ml. of distilled water should be added dropwise through the funnel. This procedure is repeated until a drop of the distillate no longer gives a precipitate when added to a dilute acetic acid solution of m-nitrobenzhydrazide. To determine the concentration of methyglyoxal, the method of Friedemann (1927) is both simple and sufficiently accurate. An aliquot is titrated with 0.1 N NaOH to a pink color with

phenolphthalein. Neutral 5% H_2O_2 solution is then added followed by a known amount of 0.1 N NaOH. (The amount will be determined by the methylglyoxal concentration. As a guide, the solution should remain pink to phenolphthalein while standing for 10 minutes at room temperature.) The flask is stoppered and allowed to stand at room temperature for 10 minutes. The solution is then titrated with 0.1 N HCl until colorless.

3) 2% sulfosalicylic acid.
4) 95% N_2 - 5% CO_2 gas mixture.
5) 0.2 M Sodium bicarbonate.

Procedure: A standard curve is prepared by adding known amounts of reduced glutathione to the yeast-methyl glyoxal system. The levels of gluthathione suggested are 0.025, 0.05, 0.1 and 0.15 mg. A blank with no glutathione is run and subtracted from these values. The flasks are set up to contain the following in the main compartment: 0.15 ml. of a 15-20 per cent yeast suspension; (The amount of yeast to be used is determined by its glyoxalase activity. This is estimated by measuring the amount of CO_2 found in 20 minutes in the presence of 0.1 mg. of glutathione and 10 mgs. of methyl glyoxal. An amount of yeast should be taken which will yield 200-250 µl. of CO_2 under these conditions); 10 mgs. of methyl glyoxal (usually about 0.5 ml.); 0.4 ml. of 0.2 M sodium bicarbonate; H_2O to make 2 ml. The sidearm holds the glutathione containing solution. If deproteinization is required, this is accomplished with 2% sulfosalicylic acid. The acid filtrate is neutralized before use with 0.2 M sodium bicarbonate to methyl orange. (This is best done with an aliquot).

After gassing with 95% N_2-5% CO_2 mixture the flasks are placed in a bath (25°) and the manometer fluid levels adjusted so that the level of the left hand column is between 0 and 5 mm. (This is accomplished by sucking gas out of the closed sytem after gassing and readjusting the level of the right column to 15. By this procedure the capacity of the manometer is almost doubled.) After an initial equilibration period with shaking (during which time the gas production in the control and experimental flasks should be equal) the contents of the side bulbs are tipped in. Readings are taken every 5 minutes without stopping the shaking apparatus. The first 5 minute reading is discarded since it will usually be too high due to mixing effects. Readings are taken for 20 minutes and the first 5 minute value calculated by extrapolation.

Calculation: The glutathione concentration is determined by reading the 20-minute CO_2 value from the standard curve.

Accuracy and Specificity: The accuracy of the method is determined chiefly by the pipetting accuracy of the different reagents. According to Woodward (1935) the limit of error does not exceed 6 per cent.

This method is highly specific since no naturally occurring sulfhydryl compound other than glutathione will react in this system. Further, ascorbic acid is not active and thus the procedure can be used in the presence of this reducing substance. While the method does not lend itself to a routine use, it is most valuable where a quantitative estimation of glutathione in biological material is required.

d-AMINO ACIDS

The determination of d-amino acids ("non-natural") is of some interest since they have been shown to occur naturally in certain bacteria. While the more classical methods of determining optical rotation usually can be used for characterizing the d-amino acids, occasionally, due to the small quantity of material available, the use of a specific enzyme preparation may be necessary for establishing the presence or the amount of these substances. (See Lipmann, et al., 1940, and Lipmann, et al., 1941). For this purpose the use of simple aqueous extracts of acetone dried kidney powder (Krebs, 1935b) is usually satisfactory. Should a pure reconstituted flavin-adenine-dinucleotide-protein system be required, the resolution and reconstitution technique of Warburg and Christian (1938) can be employed. The specificity of the two preparations is the same (Klein and Handler, 1941). For the details of preparation and procedure, the reader is referred to the original papers.

DIPHOSPHO-PYRIDINE-NUCLEOTIDE (D.P.N., COENZYME I, COZYMASE)

Two manometric methods have been used in estimating D. P. N. The first method is that reported from von Euler's (1936) laboratory and is based on the fact that the rate of yeast fermentation is dependent, within certain limits, on the D. P. N. concentration. More recently, Axelrod and Elvehjem (1939) have reported on the details of this technique as applied to animal tissues. A second method is that proposed by Jandorf, Klemperer and Hastings (1941). The principle of this method is based on the enzymatic conversion of hexosediphosphate into phosphoglyceric and glycero-phosphoric acids. The rate of this conversion is dependent within certain limits on the amount of D. P. N. present. The reaction is carried out in a CO_2 bicarbonate medium and the CO_2 formed due to the formation of the above acids is measured manometrically. The advantages claimed for this technique over the former are:

1) The reaction is dependent on fewer enzymes and in particular, does not require the presence of the unstable carboxylase system;
2) Fewer and more readily available components, which are more stable, are required;
3) The need for almost daily standardization of reagents is avoided.

However, in practice, the choice of one or the other method will be determined by the experience of the investigator, the availability of the required preparations, etc. While the writer has had no actual experience with either method for purposes of assay, it would seem that the latter method is in principle a more sound one. The reader is advised to refer to the original papers for details.

MANOMETRIC ESTIMATION OF ENZYME ACTIVITY

Since many enzymes catalyze reactions which directly or indirectly involve the production or utilization of a gas, manometric methods have been extensively used in both assaying for enzyme activity, and in studying the kinetics of a given reaction. For obvious reasons, manometric methods have been most extensively employed in the study and assay of respiratory and other oxidation enzymes. The features of the manometric techniques used for enzyme study are:

1) The need for only small amounts of enzyme preparation,
2) The accuracy and speed of the estimation, and
3) The ease with which one can study the effects of inhibitors, substrate concentration, pH, and other aspects of kinetics.

A detailed discussion of the preparation and manometric study of different enzyme systems is beyond the scope of this chapter. The reader is referred to Green (1940) and Sumner and Somers (1943) for compilations of methods and details.

Since the principles and practice of manometry have been discussed in this volume, this technique should be applicable to any enzyme system which catalyzes a reaction which directly or indirectly involves the uptake or the production of a gas.

MANOMETRIC DETERMINATION OF THIAMINE

H. F. Deutsch

Principle: (Atkins, et al., 1939). Under anaerobic conditions, the production of CO_2 by baker's yeast is stimulated by thiamine. Within certain limits this increase in CO_2 production is a linear function of the thiamine present. The method lends itself to regular Warburg apparatus (Atkins, et al., 1939) or to the larger fermentation apparatus (Schultz, et al., 1942).

Procedure: An accurately prepared suspension of baker's yeast, (Fleischman's low Vit. B_1 yeast) is used. Two mgs. of wet yeast per flask allows for an assay range of from 0.001 to 0.020 g. of thiamine. At zero time the yeast in suspension is added to a mixture of a glucose - salts, nutrient solution containing a citrate - phosphate buffer. One ml. of the above mixture is then added to the different experimental flasks containing water (blank), thiamine controls and the material to be assayed. The final substrate volume is usually 2 or 3 ml. and duplicate flasks are preferably used. The flasks are connected to their manometers and gassed with nitrogen prior to or just after placing them in a thermostat at 30°C. One hour after addition of the yeast to the nutrient-buffer

solution, initial manometer readings are taken and gas production is then measured for one hour. This hour measurement period may be divided into two or more consecutive readings if necessary. The amount of stimulation is then determined by subtracting the average CO_2 values of flasks containing no stimulatory substances from the thiamine controls and the flasks containing the assay material. The Δ thiamine value so obtained may be applied to the unknown Δ value to compute its thiamine content.

Specificity: A shortcoming of this thiamine assay method is the stimulatory activity of the pyrimidine portion of the vitamin B_1 molecule. This substance appears to be rather widely distributed in biological material in a free form. The standard procedure designed to circumvent this difficulty is to determine the activity of an unknown sample before and after treatment with Na_2SO_3. The residual stimulation after such treatment is supposedly due to the pyrimidine originally present in the sample and the difference between the two values is taken to indicate the thiamine present. However, it has recently been shown (Deutsch, 1943) that some of the stimulatory activity remains after complete splitting of thiamine with Na_2SO_3. Furthermore, pyrimidine and cocarboxylase exert varying degrees of stimulation depending on their concentrations. The variable effect of cocarboxylase can be removed by phosphatase action prior to assay. Under properly controlled conditions and with materials having low pyrimidine content, the method is more sensitive and less time consuming than the usual chemical methods.

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Chapter Fifteen

METHODS FOR THE ANALYSIS OF PHOSPHORYLATED INTERMEDIATES

G. A. LePage and W. W. Umbreit

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Chapter Fifteen

METHODS FOR THE ANALYSIS OF PHOSPHORYLATED INTERMEDIATES

That phosphorylated carbohydrate intermediates and related compounds play indispensable roles in intermediary metabolism of animal (and certain other) tissues is now well established (Lipmann, 1941; Kalckar, 1941). In recent years a great variety of analytical techniques have become available for the measurement of the individual components of tissues concerned in phosphorylative glycolysis. But since the time of Lohmann (1930) there have been few attempts to provide methods for the accurate determination of all of the compounds involved in phosphorylative glycolysis on the same sample of tissue. Yet if one wishes to study phosphorylation in intact tissues a "distribution method" of some sort is necessary. The compounds in which one is interested in such studies include the following: inorganic phosphate (ortho), total phosphorus, adenosine-tri-phosphate (ATP), adenosine-di-phosphate (ADP), adenylic acid (AA), fructose-1-6-diphosphate (hexosediphosphate), glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, phosphoglyceric acids, phosphopyruvic acid, triose phosphates (phosphoglyceraldehyde and dihydroxyacetone phosphate), phytic acid, glycogen, lactic acid, and coenzymes. Contemporary concepts of the interrelations between these materials have been reviewed by Lipmann (1941), Kalckar (1941), Burk (1939), Barron (1943), and others.

The method described below is capable of estimating each of the materials listed above on a relatively small sample of tissue (1-5 grams wet weight depending upon its phosphorus content; 5-25 mg. organic phosphorus) providing interfering substances are not present in great quantity. This depends upon the tissue one is using. The method as described is satisfactory for muscle, heart, brain, kidney, and with modifications, liver tissue of the rat, similar organs of the rabbit, for yeast and for many bacteria. It is, however, not capable of identifying all of the phosphorylated compounds which occur in the algae (Chlorella) (Emerson, Stauffer and Umbreit, 1944), in the developing oat seedling (Albaum and Umbreit, 1943), in photosynthetic bacteria, and in the anaerobic bacteria (Clostridia). One may conclude that the phosphorylated compounds in these latter tissues are different from those found in muscle and yeast. For the most part, however, especially for animal tissues the method is satisfactory.

It must be recognized that in new experimental circumstances or in other kinds of tissue, modification of the procedure may become necessary. It contains, as now designed, no provision for estimating the phosphogluconic or phospho-ribonic series of compounds which are not particularly suited to separation by barium salts. However the scheme of analysis provides a basis from which one can expand to meet new experimental needs.

The method consists, in brief, of an intermittent extraction of the "acid soluble" phosphorus with trichloracetic acid, a separation of this extract into three well-defined fractions, and the determination of the known components of these fractions by means of their characteristic properties. When this has been done, a balance is made to determine how much of the phosphorus has been accounted for in terms of known compounds. This serves to check on the accuracy of the determinations and may also serve to detect new phosphorylated compounds especially where "unaccounted for" phosphorus is to be further investigated.

METHODS OF EXTRACTION FROM TISSUE

The ease of extraction of these compounds varies greatly with the type of tissue. In animal tissues cold trichloracetic acid will usually extract the phosphorus esters and lactic acid. It is advisable to use 10% trichloracetic acid in the first extraction in order to compensate for the dilution of the acid by the tissue; further extractions to remove the material soluble but held in the tissue (by reason of its volume and liquid holding capacity) can be carried out with 5% trichloracetic acid. If animal tissue, muscle for example, is homogenized (Potter and Elvehjem, 1936, see Chapter 9) in the trichloracetic acid, the extraction takes only a few minutes and further contact with the acid for several hours does not appreciably increase the acid extractable phosphorus. However, with bacterial cells, it is usually necessary to treat the cells with approximately their own wet volume of acetone (or employ some other means of breaking down the cell) before adequate extraction of the acid soluble phosphorus is obtained. Even under these circumstances it often takes from two to five extractions of several hours each to

remove all of the acid extractable phosphorus. Such prolonged extractions must be made in the cold to avoid hydrolyzing some of the labile materials. As a general procedure one extracts the tissue, then re-extracts the residue, repeating this process until no appreciable amount of phosphorus is obtained in the last extract. In each case it is necessary to be sure that the extract obtained contains all of the acid soluble phosphorus.

In experiments with animal tissues, removal of the tissue under anaesthesia may result in rapid changes which cannot be prevented. The use of freezing techniques appears to be more satisfactory (Davenport and Davenport, 1928; Kerr, 1935). Freezing the whole animal in liquid air, dissecting the tissue while frozen, powdering it in the same condition, and adding the frozen powder to trichloracetic acid in a weighed tube, has been a satisfactory method of operation in experiments on rats. The tube is reweighed (giving the weight of tissue), homogenized, and the tissue residue centrifuged out. If the freezing is done without anaesthesia, marked changes occur due to reaction of the tissues to sudden contact with the liquid air (muscle contraction, for example). Resting values for lactic acid, high ATP, etc., are obtained if the rats are given nembutal (5 mg./100 gm. intraperitoneally) and frozen as soon as they reach light surgical anaesthesia (2-4 min.).

Dissection is carried out in a cold room (0°C.) with chisel and hammer. With practice, one can obtain the whole kidneys, whole brain, etc. Care must be taken with brain and muscle samples not to include any bone with the sample.

The tissue sample is thrown into a steel cylinder chilled with liquid air, hammered several times with a heavy piston, and the powder chilled with liquid air again. As soon as the air has just boiled off, the powder can be transferred to a paper sheet and into the trichloracetic acid without thawing until it is in the acid.

One must work rapidly from this point until the labile esters, (especially phosphocreatine) have been determined.

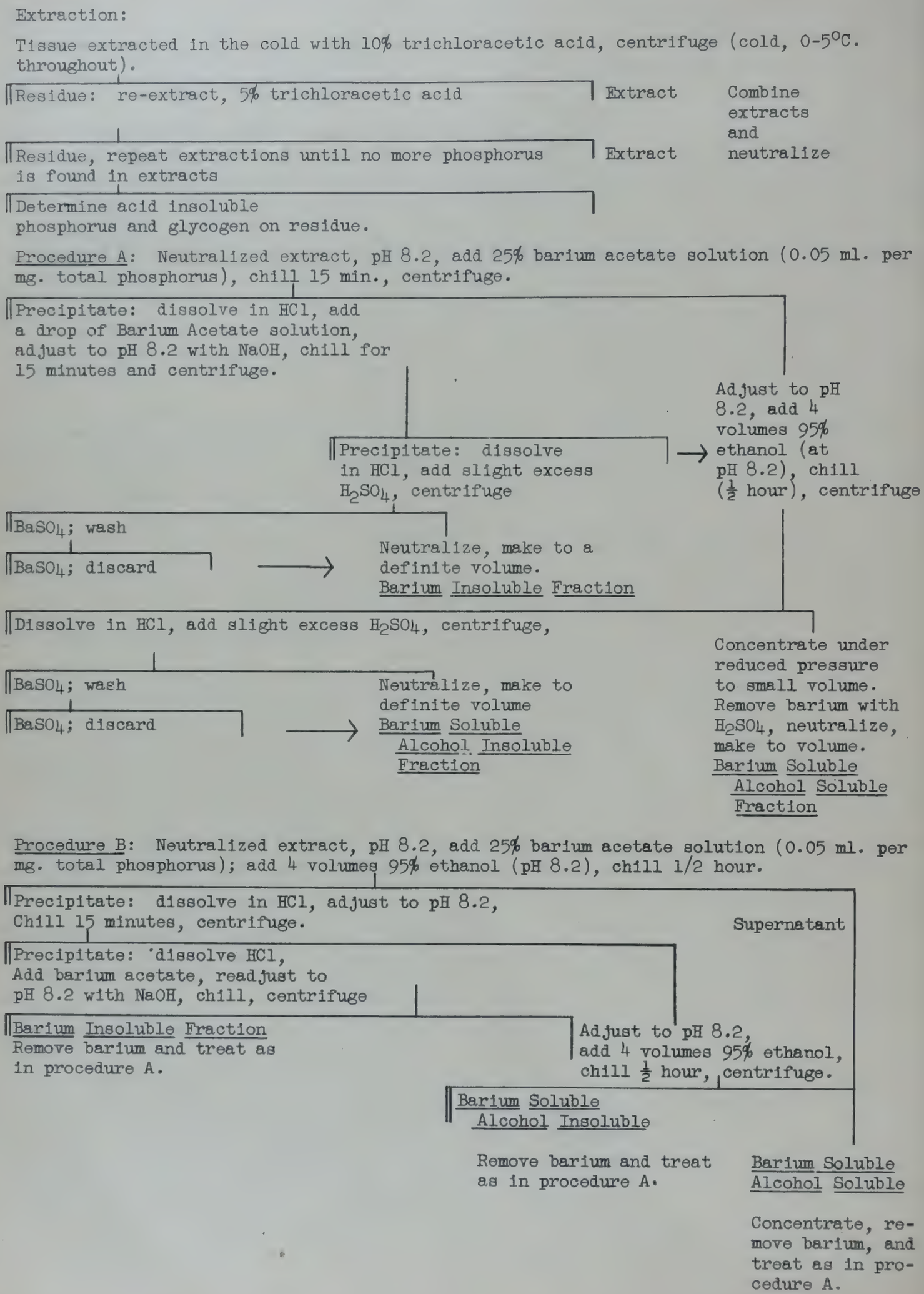
FRACTIONATION OF THE EXTRACTS WITH BARIUM

Separation of the acid soluble phosphorus compounds into three fractions, two of which contain known esters, can be accomplished by the use of the solubilities of the barium salts at pH 8.2. For such a fractionation, it is advisable to employ material containing a total of 5-25 mg. of phosphorus and to keep the original volume of the extract down to 10-50 ml. One uses concentrated solutions of reagents for the main part of the neutralizations, etc., to prevent appreciable increase in the volume of the fractions. The pH is easily adjusted to 8.2 by the addition of 1/300 volume of 1% phenolphthalein and addition of NaOH to a just discernible pink. (It is quite important that the pH is at 8.2 throughout the precipitation and variations of 0.5 pH unit on either side will cause the fractions to be contaminated with compounds which would otherwise occur elsewhere.) A slight excess of barium is added in the form of barium acetate. For example, each milligram of total phosphorus present theoretically would require 8.2 mg. barium acetate or 0.033 ml. 25% barium acetate solution. Actually for each mg. of phosphorus 0.05 ml. 25% barium acetate are added.

After the addition of the barium acetate there are two procedures which may be followed depending upon the nature of the tissue. If the trichloracetic acid has extracted a relatively large amount of polysaccharide from the tissues, the second procedure (B) is usually more suitable. Procedure A, however, is generally more convenient and is entirely satisfactory in most cases. These procedures are diagrammed in Fig. 44 and described as follows:

Procedure A: The extract after the addition of barium (at pH 8.2) is chilled in a refrigerator for 15 minutes and the precipitate is centrifuged down. The precipitated material is not homogeneous, however, and contains, in addition to the "barium insoluble" compounds, some of the "barium soluble" material in the form of double salts (Cori and Cori, 1932). These can be removed by redissolving the precipitate and reprecipitating it as follows: The crude "barium insoluble" precipitate is dissolved in 0.1 N HCl, a drop of barium acetate solution added, and the pH adjusted to 8.2 with NaOH. The suspension is chilled 15 minutes and centrifuged. The supernatant contains the "barium soluble" materials which had been precipitated with the "barium insoluble" compounds and is added to the original "barium soluble" fraction. The precipitate contains the "barium insoluble"

Fig. 44. Methods of Extraction and Fractionation



materials. It is made acid with HCl, the barium removed by the addition of a slight excess of H_2SO_4 and the barium sulfate removed by centrifugation. The barium sulfate is washed with water by centrifugation and the washings added to the "barium insoluble" fraction. This is neutralized, made to a definite volume, and held for analysis. The compounds contained in it (see Table XXV) are definitely "barium insoluble" (at pH 8.2) and further reprecipitation usually entails no loss.

The "barium soluble" fraction (including the soluble materials from the reprecipitation of the "barium insoluble" portion) is treated (pH 8.2) with 4 volumes of 95% ethyl alcohol, chilled for one-half hour, and the precipitate removed by centrifuging. It is important that it be cold since if the precipitate is taken at room temperatures the coenzymes are likely to be lost (Warburg and Christian, 1936). The precipitate is treated with HCl and a slight excess of H_2SO_4 to remove the barium as the sulfate, the $BaSO_4$ removed by centrifugation, and washed with N/100 HCl. The supernatants and washings are neutralized, made to a definite volume, and held for analysis. This constitutes the "barium soluble-alcohol precipitable" fraction (see Table XXV).

The "barium soluble-alcohol soluble" portion is concentrated under reduced pressure, at 30-35°C. to small volume and held for analysis. The compounds present in this fraction are not known. In most tissues it contains only a small proportion of the phosphorus, but in some tissues, especially plants, it may comprise as much as 30-40% of the acid soluble phosphorus.

Procedure B: This is essentially similar to procedure A except that the "barium insoluble" and "barium soluble" fractions are precipitated together by the addition of alcohol in the first step. This tends either to remove, or to dehydrate, interfering polysaccharide materials (starches or bacterial gums) which sometimes act as protective colloids and prevent a clear separation of the compounds by procedure A. The diagram (Fig. 44) is self-explanatory. The best index as to when this method should be used is the inorganic phosphorus content of the "barium soluble" fraction. If it is zero by procedure A, the separation is probably satisfactory; if inorganic phosphorus appears in any quantity in any fraction except the "barium insoluble", procedure B should be used. There is an additional modification necessary for application to rat liver which will be described later.

BASIC METHODS FOR THE ANALYSIS OF THE FRACTIONS

The fractions obtained by the procedures outlined above are analyzed for the components known to occur in them by means of certain characteristic properties of these compounds. This entails the use of a series of micromethods. The ones we have employed are listed below. Obviously these can be modified and other procedures substituted at the convenience of the investigator. For all the methods described below we have employed the Evelyn (1936) photoelectric colorimeter using slightly smaller standardized tubes (152 by 19 mm.) with an adapter so that they would fit the instrument. If the usual size Evelyn tube is used, the same quantity of reagents, etc., may be employed but the range of the methods will be slightly less. In standardizing these methods we first set up standard curves using pure compounds. All methods follow Beer's law over the range indicated and are not subject to appreciable fluctuation. Each series of analyses includes a standard from which the contents of the samples are calculated and this standard must agree with the standard curve within the limits of precision of the method. The precision quoted is the standard deviation from the mean of a series of duplicates.

Inorganic (ortho) phosphorus, including phosphocreatine: This is determined after the method of Fiske and Subbarow (1925) as follows: The sample is mixed in a colorimeter tube with 0.4 ml. 10N H_2SO_4, 0.8 ml. 2.5% ammonium molybdate, and 0.4 ml. of Fiske-Subbarow reducing agent (materials added in the order named), made to 10 ml. with distilled water. The color is developed at room temperature for 1/2 to 1 hour, or if a standard is run with each set, color development for 20 minutes is sufficient. The color is measured with the 660 mμ filter. Range, 4-40 micrograms phosphate-phosphorus. Precision, ± 0.2 micrograms.

In samples containing inorganic phosphate but no phosphocreatine, ten minute color development is sufficient. When phosphocreatine is to be determined as well, the sample should be incubated with the acid and the molybdate for twenty minutes before the addition

TABLE XXV

Chemical Properties of the Phosphorylated Esters and Related Compounds

Fraction	Compound	Method of Determination	Hydrolysis 100°C 1N HCl Per cent hydrolyzed[1] in 7 min.	180 min.	Reducing value to Folin-Malmros method	Molar Ratio Nitrogen: Pentose: Phosphorus
Barium Insoluble	ATP	N, pentose	66	-	0	5 1 3
	ADP	N, pentose	50	-	0	5 1 2
	Hexose-diphosphate	fructose	26.5	-	9.5	- - -
	Phosphoglyceric acids	specific method	0	2	0	- - -
	Phytic acid	specific method	0	0	0	- - -
Barium Soluble	Glucose-1-PO_4	hydrolysis, reduction	100	-	0 (66.4 after hydrolysis)[2]	
Alcohol Insoluble	Glucose-6-PO_4	initial reduction	-	10.5	13.2	
	Fructose-6-PO_4	reduction fructose	-	74	31.65	
	Phosphopyruvic acid	Iodine	46	-	0	
	Triose PO_4	Alkaline hydrolysis	-	-		
	Adenylic acid	N, pentose	-	58.7	0	5 1 1
	Coenzyme I	N, pentose	-	58.7	0	7 2 2
	Coenzyme II	nicotinic acid, as CoI	-	-	0	7 2 3
	Phosphocreatine	non-Ca ppt. "inorg." P	100	-	0	3 0 1
	Pentose PO_4	pentose	-	58.7	19.75	0 1 1
	Inosinic acid	N, pentose	-	58.7	0	4 1 1
Glycogen	glycogen	reduction after hydrolysis usually on extracted tissue				

[1]Hydrolysis rates of resistant esters are affected by the level of inorganic phosphate present; those of easily hydrolysable esters are not so affected.

[2]69.2% theoretical, 66.4% found.

of the reducing agent, to permit the hydrolysis of the phosphocreatine.

Precautions should be taken to exclude contamination of the extracts or reagents with silica from homogenizers or from alkaline reagents stored in soft-glass containers. With the concentrations of acid employed in the determination, silica will react as inorganic phosphorus.

Reagent: Fiske-Subbarow reducing reagent (1925): Grind 0.5 grams of sodium 1-naphtol-2-amino-4 sulfonate in 15% $NaHSO_3$, make to a volume of 195 ml. with 15% $NaHSO_3$ and add 5 ml. 20% Na_2SO_3. Warm until the materials are all in solution. Stable for several weeks if well stoppered to prevent loss of SO_2.

The value obtained by this method is a measure of both the ortho-phosphate and the phosphocreatine phosphorus since the latter is very rapidly decomposed by the molybdate reagent. Phosphocreatine is not found in yeast, plant, or bacterial tissues so far studied, hence in these tissues the method above measures inorganic phosphorus. In animal tissues, however, the apparent inorganic phosphorus is composed of two parts, the "true" inorganic phosphorus and the phosphocreatine phosphorus. These are determined as follows:

"True" inorganic phosphorus: The method is adapted from that of Fiske and Subbarow (1929). To the neutralized sample add one fifth of its volume of 10% $CaCl_2$ saturated with $Ca(OH)_2$ (at pH 8.8). Let stand at room temperature for 10 minutes. Centrifuge the precipitate and wash with a small volume of water containing 20% of the $CaCl_2$ reagent. The "true" inorganic phosphorus is thus precipitated; the phosphocreatine remains in solution. The washed precipitate is dissolved in dilute HCl and inorganic phosphorus determined on it as described above (calcium not removed). This value is the "true" inorganic phosphorus of the tissue. According to Stone (1943) calcium ATP is hydrolyzed by calcium hydroxide. In the method described above the values for both "true" inorganic and phosphocreatine are identical when the calcium precipitation is conducted at 0°C. or at 25°C. indicating that ATP is not measurably hydrolyzed during the time required (10 min.) for this precipitation.

Phosphocreatine: This is the difference between the apparent inorganic phosphorus and the "true" inorganic phosphorus, i.e., the phosphorus determined as "inorganic" by the method but which is not precipitable by calcium at pH 8.8. Since phosphocreatine is very labile in acid solution one must determine phosphocreatine as soon as possible after the extraction and keep all extracts cold until the aliquot for phosphocreatine has been neutralized. In trichloracetic acid phosphocreatine will be approximately 25% hydrolyzed in one hour at 15° C. A convenient means of avoiding use of a cold room is to keep the tubes immersed in a beaker of crushed ice until the phosphocreatine analyses are completed.

Total Phosphorus: This is estimated by digesting the sample at 100-120°C. in an oven or sand bath with 0.4 ml. 10N H_2SO_4 for 4-12 hours, depending on the amount of organic material present. After digestion, the tubes (pyrex test tubes are employed) are heated on a Kjeldahl rack with 1-2 drops of 30% H_2O_2 for a few minutes to decompose residual organic matter. The heating should be continued for a few minutes, with a few added drops of water, to destroy the peroxide but not to the extent of forming fumes from the acid. The sample is diluted with water, heated at 100°C. for 10 minutes (to hydrolyze pyrophosphates) and inorganic phosphorus determined as above without the addition of further acid. Range and precision as for inorganic phosphorus.

Reducing value: Determined by the method of Folin and Malmros (1929) in which the sample is made to 4 ml. in a colorimeter tube, 2 ml. of 0.40% $K_3Fe(CN)_6$ and 1 ml. of carbonate-cyanide mixture added. The sample is mixed, heated 8 minutes in a boiling water bath, cooled 1-2 minutes and 5 ml. ferric iron solution added to produce the color. The volume is made to 25 ml. with distilled water, mixed, and the color read using a 520 mμ filter. Range, 10-100 micrograms glucose equivalents. Precision, ± 0.4 micrograms. By reducing the volumes the method can be made more sensitive. Reagents: see Chapter 10.

This method has two advantages over methods of measuring the reducing value previously employed on phosphorylated esters. It requires very small quantities of materials and operates in a slightly alkaline solution with a very short heating time. Therefore labile materials (such as ATP or glucose-1-phosphate) do not show reducing values, as they do when more drastic methods are used. Reducing values for the esters are listed in Table XXV.

Fructose: This is determined after the method of Roe (1934). The sample is made to 2 ml. in a colorimeter tube. 2 ml. 0.1% resorcinol in 95% ethyl alcohol and 6 ml. 30% HCl are added, mixed, heated for 8 minutes in a water bath at 80°C. to develop the color, cooled and read in colorimeter with 490 mμ. filter. Range, 10-100 micrograms fructose. Precision, ± 0.4 micrograms.

It is necessary to have either pure fructose, pure fructose-6-phosphate, or pure fructose-1-6-diphosphate as a standard. Methods of preparing the fructose phosphates are described in the literature (DuBois and Potter, 1943; Neuberg and Lustig, 1942b; Neuberg, Lustig and Rothenberg, 1943). Fructose-1-6-diphosphate may be obtained from the Schwarz Laboratories, N. Y., in a rather pure form. The color obtained per micromole of hexose diphosphate is 52.5% (fructose-6-phosphate, 60.5%) that obtained per micromole pure fructose. If fructose is used as standard this must be taken into account (see determinations of hexose-di-phosphate and fructose-6-phosphate). The resorcinol solution is stable for at least a month. As far as is known the method is specific for fructose on such extracts although other keto compounds (perhaps ketogluconic acids, etc.) might conceivably interfere were they phosphorylated and able to enter these fractions.

Pentose: This is determined by the method of Mejbaum (1939). Dilute the sample to 3 ml. in a colorimeter tube; add 3 ml. of 1% orcinol in 0.1% $FeCl_3$ dissolved in conc. HCl, heat in a boiling water bath for 20 minutes. Cool, read in colorimeter with a 660 mμ. filter. Range, 4-40 micrograms pentose. Precision, ± 0.4 micrograms. This method determines pentose in ATP, ADP, adenylic acid, and coenzymes as well as in the free form. A standard of xylose, arabinose, or ribose can be used, since all three give the same value (Schlenck, 1942). The 0.1% $FeCl_3$ in concentrated HCl can be kept as a stock solution but the orcinol should be added just before the determination is made (10 mg./ml.).

Nitrogen: The sample is digested on a sand bath (150-170°C) with 1 ml. 5 N H_2SO_4 (containing 150 mg. copper selenite per liter) for 12 hours or longer. Heat over a microburner with 1-2 drops 30% H_2O_2 until fumes develop. Cool, wash into colorimeter tube, make to 10 ml. with distilled water. Add 5 ml. 2 N NaOH and 2 ml. modified Nessler's reagent. Mix, read after 10 minutes in the colorimeter using a 520 mμ. filter. Range, 10-100 micrograms of nitrogen. Precision, ± 1.5 micrograms. When relatively large quantities of organic matter are present a longer digestion is necessary. See Chapter 10 for details.

Hydrolysis curves: These are based upon the method of Lohmann (1930) and serve to roughly estimate the types of compounds in each fraction. Samples of each (neutralized) fraction are placed in tubes fitted with reflux bulbs (or stoppers) to prevent evaporation, mixed with an equal volume of 2 N HCl and placed in a vigorously boiling water bath. The tubes are removed and chilled in an ice bath after 7, 15, 30, 60 and 180 minutes of heating. Inorganic phosphorus is determined by adding (after the tubes are cold), 0.4 ml. 10 N H_2SO_4, 0.8 ml. 2.5% ammonium molybdate and 0.4 ml. Fiske-Subbarow reducing reagent as described in the method for inorganic phosphorus above. The HCl may be neutralized by the addition of 2N NaOH but it is usually more convenient to leave the acid unchanged and compare the color to standards containing an equivalent quantity of HCl. The inorganic phosphorus method is so chosen that the addition of small amounts of HCl do not significantly alter the color production, since HCl or H_2SO_4 are equivalent in the colorimetric method.

ANALYSIS OF THE BARIUM INSOLUBLE FRACTION

This fraction contains ATP, ADP, fructose-1-6-diphosphate, phosphoglyceric acids, phytic acid and inorganic phosphorus. The hydrolysis curve gives a rough index of the amounts of these materials present. They can, however, be identified with more certainty by application of the methods described below:

Inorganic phosphorus: as described.

Adenosine-tri-phosphate (ATP): This compound contains two labile phosphates (released in 7 minutes at 100°C. in 1 N HCl) and the usual method for its estimation depends upon this fact (ATP-P = Δ_7P x 3/2). But it is also the only compound in the fraction (except ADP) which contains both nitrogen and pentose. Hence estimation of these should give a N/pentose/Δ_7P ratio of 5/1/2. If ADP is the only compound present this ratio would be 5/1/1 but usually this compound is absent. If there is any question of a mixture

of ATP and ADP, enzymatic methods may be employed (Colowick and Kalckar, 1943; Kalckar, 1943) although the absence of other pentose compounds in animal tissues so far investigated would seem to make possible a calculation of the two from the above ratios, providing the nitrogen pentose ratio is 5. By means of an hydrolysis technique it is also possible to determine the nature of the ribose phosphates of the ATP molecule and thus to determine the type of ATP, i.e., adenosine-5-triphosphate providing ATP (or the corresponding ADP) are the only phospho-pentose compounds in the fraction (LePage and Umbreit, 1943).

The barium salt of ADP is more soluble than that of ATP. However, in the presence of inorganic phosphorus and other esters, its solubility is low, being only 3.2 micrograms (free ester) per ml. This was determined by freeing a trichloracetic acid extract of liver from nucleotides by precipitation with mercury at pH 5. The mercury was removed from the filtrate (with H_2S), which was then divided into two portions. One of these received ATP and inorganic phosphorus; the other received ADP in addition. Fractionation was conducted as described by procedure A. Estimation of the pentose in the resulting barium soluble fractions showed a negligible solubility of ADP (3.2 microgram/ml.).

Fructose-1-6-diphosphate (hexose-di-phosphate): This may be measured by determining the fructose in the fraction since it is the only barium insoluble compound which contains fructose. Because hexose-di-phosphate does not react as an equivalent weight of fructose, and gives only 52.5% the fructose measurement it theoretically should, the factor 3.60 is used to convert fructose measured to hexose-di-phosphate present, if pure fructose is used as a standard. If hexose-di-phosphate is used as the standard no factor of this sort is necessary. The reducing value (9.5% that of an equal weight of glucose) provides a second estimate of its amount since it is also the only compound in the fraction which is reducing. Calculation of the hexose-di-phosphate content from the two estimates should agree closely. Usually this material is only a very small part of the phosphorus of the fraction. It is 26.5% hydrolyzed by 1N HCl at 100°C in 7 minutes which serves to correct the Δ_7 value for the estimation of ATP by hydrolysis. In the presence of the inorganic phosphate levels usually found in tissues, even a few micrograms of this ester are quantitatively precipitated with barium (pH 8.2). However, in the complete absence of inorganic phosphate (the pure ester), under the same conditions (pH 8.2, excess barium) it has a rather high solubility of 2.84 mg./ml.

Phosphoglyceric acid: This is determined after the method of Rapoport (1937) as follows: Evaporate the sample to dryness in an open tube by heating in a boiling water bath. Heating in an oven or on a hot plate causes decomposition of some of the other materials and interferes with the determination. Add 2 ml. of a freshly prepared 0.1% solution of α-naphthoresorcinol in conc. H_2SO_4 and heat for 1 hour in a boiling water-bath. Cool, dilute to 10 ml. with concentrated H_2SO_4, measure in colorimeter with a 660 mμ. filter. Range, 10-80 micrograms phosphoglyceric acid. Precision, ± 0.4 micrograms.

This method is reasonably specific for phosphoglyceric acid in the barium insoluble fraction. However, Neuberg and Lustig (1943) report that the reagents do react with a wide variety of materials. Phosphoglyceric acid is resistant to acid hydrolysis, liberating only 2% of its phosphorus after three hours heating at 100°C in 1 N HCl. The value obtained experimentally must be corrected for the ribose phosphates from ATP which are only 58.7% hydrolyzed in three hours.

Of a variety of experimental conditions dealt with, some have been found (especially during drug treatment) where the colorimetric values for phosphoglyceric acid exceeded those possible from the measurement of the resistant phosphorus. In plant tissues this occurs quite frequently (e.g., Emerson, Stauffer and Umbreit, 1944). Consequently, it is necessary to use both methods on any new experimental material to determine whether the colorimetric method is reliable. A very good method based on specific rotation is described in Chapter 16. The preparation of pure phosphoglyceric acid (for use as standards) has recently been described by Neuberg (1943).

The sulfuric acid employed in the determination has some influence upon the results. C. P. sulfuric acid should be used. Some lots tend to produce a brown color in addition to the blue attributable to phosphoglyceric acid. The only solution to this difficulty that we have been able to find is to try several lots of acid until one is obtained which is free from the brown color after heating with the reagents. Studies indicate that a trace of nitric acid in the sulfuric acid is responsible for this interference.

Phytic acid: This material is not found in detectable quantity in animal tissues, or in most bacterial tissues. In seeds or seedlings it is rather high. It is very resistant to acid hydrolysis and does not react in any of the methods described for other components of this fraction, hence will appear as "unaccounted for" phosphorus when the rest of the materials have been determined. It may be estimated in two ways. First, by precipitation with calcium after the method described by Rapoport et al. (1941). However, not all of the phytate is so precipitated since there is an appreciable solubility of the calcium salt. One cannot, moreover, use the value of 85% recovery since this depends on the amount of phytate, the volume of solutions, etc. The method we have employed is to determine the recovery using samples of pure phytic acid at the same levels as might be present in the barium insoluble fraction, and to use this value to calculate, from the phytate phosphorus actually found, the amount present in the extract. In the range of 30 to 40 micrograms of phytate phosphorus per ml., the recovery of pure phytate is only 30-35%. Second: An enzymatic method, using a phytase obtained from oat seedlings or from bran is suitable. This method has recently been described by Albaum and Umbreit (1943). Both methods yield virtually identical results.

SUMMARY

Inorganic phosphorus, adenosine triphosphate (by $\Delta 7$ (corrected for hexose-di-phosphate pentose, and nitrogen), hexose-di-phosphate (by fructose and reducing value), phosphoglyceric acids (by resistant material corrected for pentose phosphates from ATP and by specific colorimetric method) and phytic acid (by precipitation or enzymatic method) can be determined upon the barium insoluble fraction. In animal and some bacterial tissues the first four account for all of the phosphorus present. If the sum of these materials do not closely approximate (90-95%) the phosphorus of the fraction, other phosphorylated compounds may be present. Under these circumstances one must search for other methods for their identification, (especially for the compounds described by Dickens (1938)) but in most tissues, especially animal tissues, estimation of the above compounds will completely account for all of the phosphorus present.

ANALYSIS OF THE BARIUM SOLUBLE - ALCOHOL PRECIPITABLE FRACTION

The compounds found in this fraction and the essential methods employed for their estimation are shown in Table XXV from which it may be noted that several of the compounds depend upon the same type of determination (i.e., adenylic acid, coenzymes, pentose phosphates), and if these materials are all present in the fraction, their determination involves additional manipulations. Glucose-1-phosphate is usually present, but in small amounts. The bulk of the material is made up of glucose-6- and fructose-6-phosphate and adenylic acid.

Phosphocreatine: As already mentioned, this is the difference between "apparent" inorganic phosphorus and that precipitated by an alkaline calcium solution. This phosphorus times $\frac{211}{31}$ = phosphocreatine. It is very rapidly hydrolyzed in the acid molybdate used in determining inorganic phosphorus. It occurs in animal tissues (muscle, brain, liver, kidney) but is not present in tissues such as bacteria, where there is no very great and sudden energy demand. Since this fraction is already free from "true" inorganic phosphorus, "apparent" inorganic phosphorus is derived from phosphocreatine. This should be checked by determining the "true" inorganic as previously described.

Glucose-1-phosphate is a relatively small component of the phosphorus in animal tissues, but is present in considerable amounts in some bacteria. It is hydrolyzed by heating in 1N HCl at 100°C. for 7 minutes and can be measured as the phosphate released in this time, correlated with the increase in reducing value (after hydrolysis, it has a reducing value with the Folin-Malmros method of 66.5% that of an equal weight of glucose) (see footnote (2) Table XXV) provided there are no hydrolyzable polysaccharides present to contribute to the reducing value. If these are present, the only method available for its estimation is the phosphorus released by 7 minute hydrolysis. (However, see glycogen). Normally not much phosphopyruvic acid will occur in intact tissues, but when the analysis for this substance by iodine titration shows appreciable amounts, correction must be made on 7 minute hydrolyzed phosphorus used to calculate glucose-1-phosphate. Phosphopyruvic acid hydrolyzes 46% in 7 min. in 1N HCl at 100°C. Its hydrolysis curve is very steep, so that care is necessary in timing the heating. Phosphocreatine also hydrolyzes completely

in the 7 minute hydrolysis but since it is also completely hydrolyzed by the reagents for determining phosphorus it does not contribute to the Δy value.

Fructose-6-phosphate: This can be measured by determining fructose on this fraction, since it is the only known compound occurring here which contains fructose. It has a reducing value, without hydrolysis, of 31.65% that of an equal weight of glucose. It is slowly hydrolyzed in acid, taking 5 hours to hydrolyze 90% in 1N HCl at 100°C. Fructose-6-PO_4 does not react with the fructose method as expected from its fructose content (fructose value is 60.5% of the theoretical), hence the factor 2.5% should be used to convert fructose found to fructose-6-phosphate, if fructose is used as the standard. With a standard of pure fructose-6-phosphate this factor is not necessary.

Glucose-6-phosphate: This ester has a reducing value of 15.5% that of an equal weight of glucose and is very resistant to hydrolysis in acid, hydrolyzing only 25-30% in 1N HCl at 100°C. in 12 hours. It can be determined as the reducing value of the fraction without hydrolysis, less the amount due to fructose-6-phosphate calculated from the fructose value, and that due to pentose phosphate not combined in nucleotides.

When these have been determined one should calculate the phosphorus accounted for by their presence. Nitrogen and pentose are determined. The ratio of N/pentose/unaccounted for phosphorus is calculated and compared with the ratios given in Table XXV (the nitrogen found is corrected for any phosphocreatine-nitrogen which may be present). This will give some indication of the type of compound for which search should be made. The flavin nucleotides are rarely encountered (except in anaerobic bacteria) and can be detected in most cases by the flavin-yellow color of the fraction. Specific enzymatic methods must be used for their assay but since they are rarely found in quantity they have been omitted here. If the "unaccounted-for" phosphorus is higher than could be accounted for by the nitrogen or pentose, the fraction is examined for phospho-pyruvic acid or triose phosphates (the latter being rarely found in any quantity in intact tissues). Methods for these materials are outlined below:

Phosphopyruvic acid: This is determined after the method of Lohmann and Meyerhof (1934) as follows: The sample is made to 3 ml. and 1 ml. of N/10 NaOH is added plus 1 ml. of N/10 iodine in KI. This is left at room temperature for 15 minutes, then made acid by addition of 0.5 ml. 1N H_2SO_4 and the excess of iodine titrated with M/100-200 $Na_2S_2O_3$. A blank is run. The iodine used went into conversion of enol-phosphopyruvic acid to iodoform. 1 ml. of M/200 $Na_2S_2O_3$ = 0.140 mg. phosphopyruvic acid. If the iodine is removed by $NaHSO_3$ and the iodoform centrifuged off, the amount of phosphopyruvic acid can be redetermined by the inorganic phosphate released, providing this is corrected for the release of phosphate from the triose phosphates. Because of the lability of this material it is desirable to determine it on the original extract as well as on this fraction. Here the inorganic phosphorus released must be used.

Triose phosphates (dihydroxyacetone phosphate and 3-glyceraldehyde phosphate): These may be determined as alkali-labile phosphorus. The sample is made to a definite volume, treated with an equal volume of 2N NaOH, left at room temperature for 20 minutes, neutralized by the addition of 2N HCl and inorganic phosphate determined. Utter and Werkman (1941) describe methods for distinguishing between the two triose phosphates but in the tissues we have examined, these compounds are rarely present in sufficient quantity to permit such distinction.

Adenylic acid: This compound will ordinarily be found in this (barium soluble) fraction, unless it is present in unusually large amounts. Under the conditions of the fractionation, barium adenylate is soluble to the extent of 11.0+ mg./ml. In some tissues, where there is not an undue amount of coenzyme present, it can be estimated by measuring pentose and nitrogen. Such analysis will give a preliminary indication of the amount of adenylic and coenzyme present in this fraction. The nitrogen and pentose found initially may agree with the ratio for adenylic acid (5 nitrogen/1 pentose/1 phosphorus) but if they do not, the next determination to be made is that for nicotinic acid (coenzymes I and II, as below), and the nitrogen and pentose values corrected for the presence of coenzymes. Enzymatic methods for adenylic acid may be employed (Schlenk and Schlenk, 1941). To distinguish adenylic acid when in a mixture of coenzymes and ribose phosphate is a difficult matter (but see "pentose phosphates" below). Unfortunately there are at present no micro methods sufficiently specific to determine adenine in these quantities.

However, a micromodification of the method of Hitchings and Fiske (1941) for total purines seems to work rather well. Even in this case the adenine figure obtained must be corrected for the adenine contained in the coenzymes present.

Pentose phosphates: These are determinable as pentose not associated with nitrogen. They tend to complicate the adenylic acid measurements, but in most tissues (animal, bacteria) do not occur in large quantity. The adenine-pentose bond, however, is very unstable and can be broken by heating for 10 minutes at 100°C. in 1 N HCl. Under these circumstances the pentose-nicotinamide bond of the coenzymes is not affected (at least for cozymase). Hence one can determine the pentose not associated with nitrogen in the following manner: A sample of the fraction is made 1 N with respect to HCl and heated in a boiling water bath for 10 minutes. This breaks all the pentose-adenine bonds, but does not destroy (i.e., less than 1%) the pentose to nicotinamide link, nor does it remove any of the phosphorus from the ribose phosphates. This conclusion was arrived at from the finding that the nicotinamide was recovered in the 10 minute hydrolyzed material precipitated with barium and alcohol. This is in direct contradiction to the finding of F. Schlenk that the nicotinamide-ribose linkage of coenzyme I hydrolyzed slightly faster in acid that did the adenine-riboside linkage. However, the coenzyme here measured is probably not a true measure of coenzyme in animal tissues and the form being dealt with is an inactivated form. The material is neutralized to pH 8.2, barium added, and treated with 4 volumes of alcohol (cold, 3 hours). This precipitates the ribose-phosphates and the nicotinamide-ribose-phosphate but the adenine remains in solution. After separation and removal of barium, the nitrogen determined in the precipitate will be that associated with nicotinamide-ribose-phosphate, while nitrogen in the supernatant results from the adenine. Any residual pentose not accounted for by association with these compounds is free pentose phosphate. In addition to determining nitrogen on these fractions, estimation of nicotinic acid (nicotinamide) on the precipitated portion permits the detection of any amino-acids which might possibly have been extracted (aspartic, glutamic acids, especially). Such amino-acids would follow the nicotinamide-ribose-phosphate rather than the adenine in the fractionation.

"Coenzymes": There are three means available for differentiating between coenzymes and the other component of the fraction which contains pentose and nitrogen (adenylic acid). The first is most direct, making use of assay for nicotinic acid, either chemically or biologically, on the fraction. The second makes use of the fact that the adenine-ribose-phosphate bond is sensitive to acid (as described under ribose-phosphate). The third is an enzymatic method (Jandorf et al., 1941; Warburg et al., 1935). Chemically we have employed the first two as described above. The nicotinic acid method we have used is a modification of that of Bandier and Hald (1939) as follows: (We are indebted to Mr. R. L. Emerson for his study of this method).

Nicotinic acid: Add to the sample an equal volume of 2N NaOH and heat in boiling water bath for one hour (this is to hydrolyze coenzymes (and nicotinamide) to nicotinic acid). Cool, neutralize with 2N HCl. Dilute to 9 ml. Heat for 5 minutes in a water bath at 78-80°C.; add 1 ml. of cyanogen bromide reagent. Heat for 5 more minutes in the water bath at 78-80°C. Cool to room temperature. Add 10 ml. photol reagent. Mix and allow to stand at room temperature for one hour protected from direct light. Read in colorimeter using 420 mμ filter. Range, 10-100 micrograms nicotinic acid. Precision, ± 0.3 micrograms.

Reagents: cyanogen bromide: To a saturated solution of bromine in water, add NaCN or KCN (half saturated solution) until just decolorized. Use soon after preparation. Prepare in hood, add to samples with burette. Photol reagent: prepare a saturated solution at room temperature by shaking 5-7 grams photol in 100 ml. water. The solution must be shaken thoroughly, protected from light, and used within 2-3 hours after preparation. The color produced is a clear yellow; if a brown color appears in the determination it must be discarded and fresh reagents prepared.

Glycogen and related carbohydrate polymers: Glycogen can be determined by complete solution of the extracted tissue in 30% aqueous KOH by heating the mixture in a boiling water-bath 10-20 minutes, precipitating with 1 to 1.1 volumes 95% EtOH while hot, then cool in refrigerator, after the method of Good et al., (1933). Centrifuge down the precipitate, discard supernatant and hydrolyze the precipitate in 1N HCl for 2 hours in a boiling water-bath. Measure the reducing sugar with the Shaffer-Somogyi copper reagent

(1933). Use the factor 0.927 to calculate anhydrous glycogen from glucose. This is satisfactory for all tissues except brain. Here it is necessary to introduce a modification. Before hydrolysis one must wash the precipitate three times with a mixture of 20 volumes $CHCl_3$ and 80 volumes methanol. This removes cerebrosides which otherwise cause error by hydrolyzing to yield reducing compounds (Kerr, 1936).

Unlike muscle, kidney, or brain from which virtually no glycogen is extracted, trichloracetic acid will remove most of the glycogen from liver (rat, rabbit). The extracted glycogen appears in the "barium soluble alcohol insoluble" fraction. It complicates the glucose-1-phosphate determination and interferes with precipitation of the barium insoluble fraction. In the case of liver extracts (the only material in which it appears) it can be removed as follows:

The trichloracetic acid extract (not neutralized) is treated with one volume of 95% EtOH and the resulting precipitate centrifuged. The precipitate is glycogen. In this acid solution the esters remain dissolved. There is some phosphorus left associated with the glycogen, but this is non-dialyzable, and so is not ester-phosphate. The precipitate here can be dissolved in water and glycogen determined. The supernatant from the precipitation must be treated with NaOH and $Ba(OAc)_2$ to adjust pH to 8.2 and a further 3 volumes of EtOH added. The precipitate is then dissolved in dilute HCl and fractionated as usual. Unless this is done, the fractions do not separate clearly because the glycogen acts as a protective colloid, preventing proper precipitation.

The separation technique described above can probably be applied to cases where bacterial gums, starch, etc. interfere with the separations and are not removed by Procedure B. The added procedure gives no loss of esters in fractions containing only the usual materials (glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, adenylic acid, phosphopyruvic acid, coenzymes and phosphocreatine).

It has been our practice to determine glycogen on the same tissue sample as was used for the phosphate ester determinations by using the trichloracetic acid extracted residue, dissolving the latter in 30% KOH, and proceeding as in the usual methods. This gives essentially the same results as the use of fresh tissue.

SUMMARY

Creatine phosphate, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, adenylic acid, coenzymes I and II, ribose phosphate, phosphopyruvic acid, and triose phosphates can be determined on the barium soluble alcohol precipitable fraction. Glycogen may be determined on the extracted tissue but sometimes appears in the trichloracetic acid extract. Usually, however, only a few of these compounds occur in any quantity in a given tissue so that the actual determinations can be considerably simplified once the composition of the tissue is known.

ANALYSIS OF THE BARIUM SOLUBLE-ALCOHOL SOLUBLE FRACTION

This fraction contains unknown materials as well as most of the salts and reagents used in the separation. Its further analysis is restricted to the determination of the total phosphorus it contains. Since the amount of phosphorus occurring in the fraction is generally small, we have made no detailed study of the compounds contained in it. It would be necessary to separate them from the salts (chiefly sodium trichloracetate) present.

Lactic acid: We determine lactic acid where necessary after the method of Barker and Summerson (1941) on an aliquot of the original trichloracetic acid extract as described in Chapter 10.

APPLICATION

An example of the application of this method for the identification of the phosphorylated compounds in a bacterial tissue has been given by LePage and Umbreit (1943a). An example of an analysis of animal tissue is described below. For this purpose we have presented representative analyses of rat liver tissue, since this is somewhat more complex than the usual distributions encountered.. Data from the analyses are given in Table XXVI.

The content of the various compounds is calculated as follows: Total acid extractable phosphorus: 5.57 x 100/5 = 111 mg. %. Inorganic phosphorus: 0.950 x 100/5 = 19.0 mg. %. Phosphocreatine: (1.317 - 0.95) 100/5 x 211/31 = 50 mg. %. Hexose-di-phosphate: 0.342 x 3.60 x 100/5 = 24.6 mg. %. Phosphoglyceric acids: 0.990 x 100/5 = 19.8 mg. %. ATP and ADP: The ratio of nitrogen to pentose is 5.17; the ratio of phosphorus released in 7 minutes to pentose is 1.07, corrected for 26.5% hydrolysis of the hexose-di-phosphate is 1.048. Since the nitrogen-pentose ratio is 5, one is justified in computing the ADP from the deficiency of the labile phosphate to pentose ratio from the value of 2 which would occur if all of the material were in the form of ATP. The ADP is represented by (2-1.048) x 100 = 95.2% of the pentose. Therefore ADP is 1.38 x 0.952 x 423/150 x 100/5 = 74.2 mg. %. ATP is 1.38 x 0.048 x 503/150 x 100/5 = 4.4 mg. %. Phosphopyruvic acid: 0.25 x 10 x 0.140 x 100/5 = 7.0 mg. %. Glucose-1-phosphate: The labile phosphorus (0.144 mg.) must be corrected for phosphopyruvate hydrolysis (46%) which is 0.03 mg. hence: (0.144-0.03) x 260/31 x 100/5 = 23.7 mg. %. Fructose-6-phosphate: 0.518 x 2.39 x 100/5 = 24.8 mg. %. Glucose-6-phosphate: (1.736 - 0.904) x 100/13.2 x 100/5 = 126 mg. %. The nitrogen of the barium soluble alcohol insoluble fraction results from phosphocreatine, adenylic acid, coenzymes (I and II) and perhaps amino acids. Phosphocreatine nitrogen is 0.286 x 42/31 = 0.387 mg. Nitrogen present as adenine (that not precipitated after 10 min. hydrolysis in 1N HCl at 100°C. followed by barium fractionation (as previously described)) is 1.432 - 0.345 - 0.387 = 0.600 mg. Coenzymes (as cozymase): 0.273 x 20 x $\frac{680}{123}$ = 30.1 mg. %. Adenylic acid: (0.600 - 0.156) x $\frac{347}{70}$ x 20 = 44.0 mg. %. This is calculated from the adenine nitrogen less the adenine due to the coenzymes. Pentose not associated with nitrogen is pentose phosphates. Pentose phosphate: (2.770 - 1.085) x $\frac{230}{150}$ x 20 = 51.7 mg.%.

It will be noted from Table XXVI that the compounds present in each fraction satisfactorily account for a large proportion of the phosphorus. The actual compounds identified constitute 82.7% of the acid extractable phosphorus. 5.3% more was associated with the glycogen fraction (in an organic non-dialyzable form) and the remainder (12.0%) was presumably (although not determined in this case) in the unknown compounds of the barium soluble alcohol soluble fraction. The increased reducing value on 2 hour hydrolysis is all accounted for by hydrolysis of adenine-ribose linkages, glucose-1-phosphate, etc., so that complete separation of glycogen from the esters was accomplished.

It will be noted that liver is indicated to contain little ATP. That this is actually the case, at least under these conditions, rather than that another pentose compound is present in the Ba-insoluble fraction, was demonstrated by isolation of ADP in 77% yield.

That other pentose compounds do not occur in the fraction in brain, muscle, kidney, heart is demonstrated by reason of having found occasions where resting tissues had ATP, no ADP, as calculated from pentose: $\triangle$ $_7$P ratios.

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(References continued on page 174)

TABLE XXVI

Analysis of Phosphorylated Esters and Related Compounds in Rat Liver

Normal rat, frozen by immersion in liquid air, liver dissected, powdered and 5 grams (wet weight) placed in trichloracetic acid. 30 ml. extract contained 5.57 mg. total phosphorus, total inorganic phosphorus 1.317 mg., "true" inorganic phosphorus 0.950 mg., 25 ml. fractionated. All data below calculated back to 5 gram wet weight liver.

Determinations	Barium Insoluble	Purified Glycogen	Barium Soluble Alcohol Insoluble	Barium Soluble Alcohol Soluble
(Volume of fraction, ml.)	(10)	(15)	(15)	(20)
	mg.	mg.	mg.	mg.
Phosphorus				
Total	1.84	0.28	2.78	(0.83*)
Inorganic	0.930	0.000	0.311	0.00
Calcium precipitable	--	--	0.025	
Creatine	--	--	0.286	
Hydrolysable (7 min.)	0.305		0.282	
Pentose	1.380		2.770	
Nitrogen	0.665		1.432	
(Molar ratio, Nitrogen/Pentose)	(5.17)			
Reducing value without hydrolysis (mg. glucose equivalent)	0.119		1.736	
with hydrolysis (100°, 2 hr)	--	127.0	3.43	
Fructose (Fructose standard)	0.342		0.518	
Phosphoglyceric acids	0.990			
Nicotinic acid			0.273	

*(This value calculated, by difference, not determined, probably should be lower because recoveries are not 100%)

Reducing value after alkali digestion on extracted tissue (glycogen not extracted by trichloracetic), 12.05 mg. glucose. Phosphopyruvic analysis on barium soluble alcohol insoluble fraction using 10% of fraction for analysis; ml. M/200 thiosulfate, sample, 19.70; blank, 19.95.

No allowance was made in this particular analysis for inosinic acid, which would produce a slight error in the adenylic acid and free pentose phosphate measurements. There is no large amount of inosinic acid in normal, resting liver. Estimation of it by difference can be achieved by use of Schlenk's (1942) enzymatic method for adenylic acid.

Hydrolysis 10 min. at 100° C. in 1N HCl and refractionation of the barium soluble alcohol insoluble fraction gave pentose recovery of 93% (2.575 mg.) and nitrogen 0.338 mg., of which 0.058 mg. was present as nicotinamide. Hence the rest of the nitrogen in the fraction (0.280 mg.) is amino acid nitrogen. In the original fraction 0.600 mg. of nitrogen must have been present as adenine (+ hypoxanthine or inosinic). Recovery of nicotinamide was also 93%. The calculation back to original barium soluble alcohol insoluble is thus $x=\frac{100}{93}$.

Compounds		mg. %[2]	In % phosphorus of fraction
Fraction	Compound		
Barium Insoluble	"true" inorganic	19 (18.6)[1]	50.5
	ATP	4.4	2.2
	ADP	74.2	29.9
	Hexose-di-phosphate	24.6	6.1
	Phosphoglyceric acids	19.8	9.0
Total			97.7

Barium	Phosphocreatine	50.0 (38.9)[1]	13.2
Soluble	Phosphopyruvic acid	7.0	2.3
Alcohol	glucose-1-PO_4	23.7	5.1
Insoluble	fructose-6-PO_4	24.8	5.3
	glucose-6-PO_4	126	27.0
	Adenylic acid	44.0	7.1
	Coenzyme (as CoI)	30.1	5.0
	Pentose phosphate	51.7	12.5
Total			77.5[3]
Glycogen	Phosphorus	5.6	
	Glycogen (total)	2601	
	extracted	2360	
	not extracted	241	

[1]found in fraction
[2]in terms of esters as free acids
[3]the bulk of the unaccounted for phosphorus of this fraction is probably phosphoglycerol, see LePage (1944).

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Chapter Sixteen

PREPARATION OF PHYSIOLOGICALLY IMPORTANT INTERMEDIATES AND METABOLITES

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Chapter Sixteen

PREPARATION OF PHYSIOLOGICALLY IMPORTANT INTERMEDIATES AND METABOLITES

INTRODUCTION

In the course of study of tissue metabolism, certain materials are required which are not available from chemical supply houses. The preparation of some of these are described in this chapter. Whenever possible synthetic methods are described on the condition, however, that we ourselves have successfully employed the syntheses described. Certain materials are obtainable only by isolation. In these, the principle followed is that the starting material should be as high as possible in the component to be isolated. Only isolations which we ourselves have successfully done are described. Some of the materials are available on the market but in rather limited amounts from rather limited sources. In these cases we have referred to the sources from which we have obtained the material without reference to whether any more of the material may be available or whether other suppliers are able to fill the need.

The material contained in this chapter is concerned only with the following subjects:

1. The preparation of the principle intermediates of the "Meyerhof-Embden System".
2. The preparation of the principle intermediates of the Kreb's "Tri-carboxylic-acid Cycle".
3. The preparation of certain important electron transporting systems and cofactors.
4. The preparation of buffers and certain widely used suspending materials.

INTERMEDIATES OF THE MEYERHOF-EMBDEN SYSTEM AND RELATED COMPOUNDS

These phosphorylated materials play a key role in tissue metabolism. Discussion of their role will be found throughout the literature but especially in the papers and reviews of Burk (1939), Cori (1942), Barron (1943), Lipmann (1941), Kalckar (1941), Meyerhof (1941, 1942), Potter (1944) and others. Methods for determining these materials are discussed in Chapter 15. The materials concerned are the following:

Material	Page	Material	Page
Glucose-1-phosphate	176	Adenosine-tri-phosphate	181
Fructose-1-phosphate	177	Adenosine-di-phosphate	181
"Embden's ester"	178	Adenylic acid	182
Glucose-6-phosphate	178	Adenosine	182
Fructose-6-phosphate	178	Adenine	182
Fructose-1-6-diphosphate	178	Inosine-tri-phosphate	182
Phosphoglyceric acids	178	Inosine-di-phosphate	183
Special determination	178	Inosinic acid	183
Phosphopyruvic acid	179	Phosphogluconic acid	184
Phosphocreatine	180	Acetyl phosphate	184
		Magnesia mixture	184

PREPARATION OF GLUCOSE-1-PHOSPHATE

J. F. Stauffer

The method of Haines (1940) of preparing glucose-1-phosphate from starch and inorganic phosphate in the presence of phosphorylase from potato tubers has been found to be satisfactory.

The enzyme preparation is obtained as follows (a purified enzyme preparation may be used if desired; Haines (1940) and Green and Stumpf (1943) both outline its preparation in considerable detail): About 1800 grams of ordinary market grade potato tubers are washed, sliced and ground to a pulp in a nixtamal mill or similar device. The juice is extracted by pressing it out through canvas or several layers of cheese-cloth in a hydraulic press. Ordinarily about 900 ml. of juice is obtained. This juice is to be used immediately in setting up the digest outlined below. If there is to be any delay in completing the above extraction, add sufficient toluene to saturate the pulp.

The digest for the preparation of the glucose-1-phosphate is prepared as follows: 75 grams of potato starch is made into a paste by slowly washing it into 4 liter of boiling water and, meanwhile stirring vigorously, holding it at 100°C. for 30 minutes, or until a smooth paste is obtained. When the paste has cooled to room temperature add, with thorough mixing, the following: 850 ml. of the phosphorylase preparation; 2.125 liters of 0.25 M, (pH 6.8) phosphate buffer (KH_2PO_4 and K_2HPO_4); 10-15 ml. of toluene; sufficient water to make the total volume 8.5 liters. Allow the reaction to proceed at room temperature for 48 hours or until the inorganic phosphate reaches a minimum (see Chapter 15 for phosphate determination). Inactivate the enzyme by heating the digest to 100°C. Precipitate most of the inorganic phosphate by adding saturated Mg-acetate solution and a slight excess of ammonia. This step is easily followed since the precipitate settles out quite rapidly. The precipitate is removed by filtration. Reduce the volume of the filtrate to 4 liters by removing water by freezing (the water may be removed by vacuum distillation), as follows: The filtrate is placed in 250 ml. test tubes in a freezing cabinet at -10 to -20°C. until approximately three-fourths of the volume of the liquid is ice; the liquid is poured off into a suitable container, and the ice is dumped onto the plate of a Buchner funnel, crushed and washed with a small amount of water (this operation is carried out rapidly and the ice pressed with a wooden disc to speed the removal of the wash water). The several washings are concentrated by freezing and added to the main portion of the concentrated liquor. The phosphate ester is converted into its barium salt by adding 85 gms. of Ba-acetate. Seven liters of methyl alcohol are added, and the precipitated Ba-salt allowed to settle for 12-15 hours, then removed by centrifuging. The Ba-salt is taken up in 1 liter of water and brought to a pH of 1.8 with H_2SO_4. The barium sulfate is removed by centrifuging, ground and extracted three times with 150 ml. portions of water, which are added to the centrifugate above. The liquid containing the ester is now adjusted to pH 8.3 with KOH and 1.2 volumes of methyl alcohol added. The crude K-salt of the ester is allowed to settle out for 24 hours and is removed by centrifuging. This salt is suspended in 1 liter of water and the pH adjusted to 5.5 with acetic acid. 200 mg. of α-malt amylase, suspended in a small quantity of water, is added and the digest allowed to stand for 2 hours. Trichloracetic acid (50%) is now added to give a final concentration of 5%, followed by 1.5 volumes of 95% ethyl alcohol. The precipitate is removed by filtration and discarded. The filtrate is adjusted to a pH of 8.3 with KOH and placed at 0°C. for 24 hours The K-salt of the ester crystallizes out. It is removed by centrifuging and dried in vacuo over $CaCl_2$. This salt is further purified by dissolving it in 1 liter of water (at 50°C.), stirring in 3 gms. of charcoal and filtering; 1.0 liter of hot acetone is then added to the filtrate. The K-salt is allowed to crystallize out at room temperature for 24 hours, followed by two days at 0°C. after which 500 ml. of acetone are added and crystallization allowed to proceed at 0°C. for 3-4 days. The precipitate is removed by centrifuging and dried in vacuo over $CaCl_2$. This procedure, according to Haines (1940) yields a salt of the following formula: $C_6H_{11}O_5 \cdot O \cdot PO_3 \cdot K_2 \cdot 2H_2O$, whose water of crystallization is lost at 1 mm. over P_2O_5 at 100°C. Additional information on the preparation of this ester will be found in papers by Cori, Colowick and Cori (1937) and Sumner and Somers (1944). Its synthesis is described by Cori, Colowick and Cori (1937).

FRUCTOSE-1-PHOSPHATE

W. W. Umbreit

This compound is not ordinarily considered to be an intermediate in the Meyerhof-Embden system, but there is some evidence to show that it may act as an intermediate between this system and bacterial polysaccharides. The method of preparation and the properties of the ester have been described by Macleod and Robison (1933), Tanko and Robison (1935) and Pany (1942). Bone phosphatase is allowed to act on hexosediphosphate until one phosphate group is removed. This is removed at either end of the hexose-diphosphate molecule yielding 50% fructose-1-phosphate and 50% of a mixture of fructose-6-phosphate and glucose-6-phosphate (the latter arising because of a phosphohexokinase in the bone phosphatase). The inorganic phosphate released and any residual hexosediphosphate is removed in the barium insoluble fraction (see analytical methods, Chapter 15) and the mixture of monophosphates is removed in the barium soluble alcohol precipitable fraction. The glucose-6-phosphate is oxidized to phosphogluconic acid (which is removed in the barium insoluble fraction) by bromine, and the fructose phosphates remaining are separated by the fractional crystallization of their brucine salts. Fructose-1-phosphate is as labile as glucose-1-phosphate to acid and is highly levo-rotatory.

"EMBDEN'S ESTER"

This is an equilibrium mixture of glucose-6-phosphate and fructose-6-phosphate. Its preparation from yeast has been described in some detail by Warburg and Christian (1932) (especially p. 456 ff.), and a convenient preparation from fresh yeast has recently been reported by DuBois and Potter (1943). The "ester" is valuable as a source of "monophosphates" for enzyme studies and as a source of glucose-6-phosphate and phosphogluconic acid.

GLUCOSE-6-PHOSPHATE (ROBISON ESTER)

W. W. Umbreit

Warburg and Christian (1932) give a detailed account of the preparation of this compound. A simple method of preparation from Embden's ester as used by LePage and Umbreit (1943) is as follows:

Hydrolyze Embden's ester in 1 N HCl at 100°C. for 12 hours. This destroys all of the fructose-6-phosphate and 20-25% of the glucose-6-phosphate present. Fractionate the extract according to the analytical separation (Chapter 15), the inorganic phosphate released appears in the barium insoluble fraction, the glucose-6-phosphate in the barium soluble alcohol insoluble fraction and the glucose and fructose released by the hydrolysis appear in the barium soluble alcohol soluble fraction. Several reprecipitations of the glucose-6-phosphate yields an analytically pure product.

FRUCTOSE-6-PHOSPHATE (NEUBERG ESTER)

This compound is prepared by the acid hydrolysis of hexosediphosphate. After hydrolysis, the inorganic phosphate and the residual hexosediphosphate are precipitable as the barium or calcium salts leaving fructose-6-phosphate in solution, from which it may be precipitated (as the barium or calcium salt) by the addition of four volumes of alcohol. Details of the method are described by Neuberg, Lustig and Rothenberg (1943).

FRUCTOSE-1-6-DIPHOSPHATE (HEXOSE-DIPHOSPHATE, HARDEN-YOUNG ESTER)

This substance is now available in the form of relatively inexpensive barium or calcium salts from the Schwarz Laboratories (202 East 44th Street, New York City) and hence no longer need be prepared by the individual investigator. While Lebedev extracts have been widely used in its preparation, Neuberg and Lustig (1942a) and DuBois and Potter (1943) have described methods using fresh yeast. Neuberg, Lustig and Rothenberg (1943) describe a simple and convenient method of preparing the pure compound (by means of its acid salts) and give a rather complete summary of its chemical properties.

PHOSPHOGLYCERIC ACIDS

W. W. Umbreit

Two compounds are of particular interest, i.e., d(-) 3-phosphoglyceric acid (Nilsson-Lohmann ester) and d(+) 2-phosphoglyceric acid (Kiessling ester). The synthesis of both has recently been described by Neuberg (1943). In tissues they are in equilibrium, with the 3-ester predominating. The preparation of the 3-ester from both fresh or dried yeast is relatively simple (Neuberg and Lustig, 1942b) and consists in allowing the fermentation to go on in the presence of fluoride and acetaldehyde. Further, the acid is readily prepared from almost any tissue. Inasmuch as it is the only compound resistant to acid hydrolysis in the barium insoluble fraction of yeast and animal tissues it may be readily isolated by subjecting the separated barium insoluble fraction to acid hydrolysis (1 N HCl, 100°C, 6 hours), precipitating the inorganic phosphate and unhydrolyzed phosphoglyceric acid with barium, removing the barium with sulfuric acid, the phosphate with magnesia mixture, and reprecipitating the pure phosphoglyceric acid with barium. (See Chapter 15.)

DETERMINATION OF PHOSPHOGLYCERIC ACID BY OPTICAL ROTATION IN THE PRESENCE OF MOLYBDATE

H. A. Lardy

This method is so clearly described in the original publication (Meyerhof and Schulz, 1938) that the only reason for describing it here is to provide the details for those to whom the original journal may not be available. The method is far more specific than that of Rapoport (see page 167); the only interfering substances being other α-hydroxy acids and excessively large amounts of inorganic phosphorus. Inorganic phosphorus may be

removed with magnesia mixture and the phosphoglyceric may be separated from most other acids by precipitation with lead acetate at a pH of about five.

The optical rotation of the acidified solution of phosphoglyceric acid is determined before and after the addition of 1/3 volume of 25% ammonium molybdate. For d(-) 3-phosphoglyceric acid in N HCl Meyerhof gives $(\alpha)_D^{20} = -13.2°$; with molybdate $(\alpha)_D^{20} = -745°$. The $(\alpha)_D$ of the naturally occurring equilibrium mixture of d(-)3-phosphoglyceric and d(+)2-phosphoglyceric acids is according to Meyerhof -650° to -670° in the presence of molybdate.

Example: The specific rotation is defined as

$$(\alpha)_D^t = 100\,\alpha/e\,c$$

Where: α = observed angle of rotation.
e = length of tube in decimeters.
c = grams of material dissolved in 100 ml. solution.
t = temperature of the experiments.
D = wave length of light used (D line of sodium).

Solution of 0.907 mg. 3(-)phosphoglyceric per ml. used (pure ester).

(no molybdate) = 0; after addition of 1/3 volume of 25% ammonium molybdate = -0.45°; length of tube = one decimeter. (21°C.).

$$C = 100\,\alpha/e\,(\alpha)_D^t = 100 \times -0.45/1 \times -745 = 0.0604$$

Thus 0.0604 grams exist in 100 ml., or 0.604 mg. per ml. This had been diluted 1/3 by the addition of molybdate, hence original concentration found was 0.604 x 3/2 = 0.906 mg./ml. found; taken: 0.907 mg./ml. Specificity permits its use in extracts without extensive purification.

ENOL-PHOSPHOPYRUVIC ACID

G. A. LePage

The procedure employed is modified from that of Keissling (1936).

Quinoline to be used in this procedure should be freed of water by treatment with CaO, and distillation under vacuum, or CaO separated and the quinoline distilled at ordinary atmospheric pressure. The pyruvic acid should be distilled under vacuum (5-8 mm., 35-40°C) and the first third of the distillate discarded to avoid including water.

20 gm. of pyruvic acid is dissolved in 60 gm. of quinoline and the mixture put in a flask, equipped with an electric stirrer, so that very efficient mixing can be obtained. The flask is immersed in an oil bath at 70°C. and a solution of 115 gm. of phosphorus oxychloride in 120 gm. quinoline added slowly over a period of 20 minutes, with rapid stirring. Stirring is continued for 5-10 minutes to complete the reaction; then the flask is stoppered and chilled in ice. During the reaction time, do not permit the pH to go below 3.

The contents of the flask are gradually added to 265 ml. of cold 50% NaOH in a beaker immersed in ice. Chopped ice may be added to the solution to keep the temperature down. If necessary, add more NaOH to make the solution just alkaline to phenolphthalein. Separate off as much as possible of the quinoline. Add an excess of barium acetate (about 560 ml. of 2M) and 2 volumes of 95% EtOH (chilled). If necessary readjust the pH to be just alkaline to phenolphthalein. Centrifuge out the precipitate and suspend it in 600-700 ml. of 0.1 N HCl. Add 162 gm. of Na_2SO_4. Stir vigorously or mix in a Waring Blendor. Centrifuge and wash the precipitate. Treat supernatant and washings with 65 gm. NH_4Cl + 250 gm. $MgCl_2$, neutralize to a phenolphthalein color and mix in the Waring Blendor. Add a few ml. of concentrated NH_4OH and let the solution stand in the refrigerator 1/2 to 1 hour. Centrifuge and discard the precipitate. Neutralize to pH 4.5 with acetic acid, add barium acetate and centrifuge off any $BaSO_4$ formed. Add an excess of barium acetate (25-35 ml., 2M) and two volumes of 95% EtOH. Adjust to pH 8.0, chill, and centrifuge the precipitate. Take up this precipitate in 300 ml. 0.1 N HCl and treat with norite in the

cold, mixing well and filtering off the norite. If the solution is not almost "water-white", add more norite. The clear solution, freed of norite, is adjusted to pH 8.0, an excess of barium acetate and 2 volumes of 95% EtOH added. The resulting precipitate, after chilling of the solution 30 minutes, is centrifuged, washed successively with 95% EtOH and ether, and dried rapidly, under vacuum, in a desiccator over $CaCl_2$.

The product (1.5-2.5 gms.) may still contain some inorganic phosphorus. If there is sufficient present to be objectionable, it can be removed by reprocessing, treating with H_2SO_4 to remove barium, precipitation of inorganic phosphorus with magnesia mixture and reprecipitation of the phospho-enol-pyruvate with barium and alcohol.

PHOSPHOCREATINE

A. L. Lehninger

This compound is most easily prepared synthetically. The synthesis of Zeile and Fawaz (1938) has been found to be reliable. This involves the direct phosphorylation of creatine with $POCl_3$.

Grind 10 grams of creatine hydrate (Eastman) thoroughly with 9 ml. of 17 N NaOH. After the mixture has been completely homogenized, dilute with 100 ml. water and centrifuge off any undissolved material. Cool the solution in an ice-bath and add, with stirring, 4 ml. of $POCl_3$ and 15 ml. of 17 N NaOH, in that order. Repeat these additions three more times adding ice-cubes to the mixture between additions, so that the final volume of $POCl_3$ added is 20 ml. and that of 17 N NaOH is 75 ml. The final volume of the reaction mixture should be about 450 ml. Allow to stand 20 minutes in an ice-bath after the last addition. The precipitate (which is Na_3PO_4) is filtered off through asbestos-glass wool. The precipitate is washed with a few ml. of water and the washings are added to the filtrate.

The filtrate is now carefully neutralized with cold concentrated HCl to a weak phenolphthalein red, while in an ice bath, with efficient stirring. From this point on the solution must not be allowed to become acid because phosphocreatine is stable only in a slightly alkaline solution. The solution is then concentrated to a volume of 150 ml. either by distillation *in vacuo* at 25°C. or by blowing clean, dry compressed air over the surface of the solution in a large evaporating dish. The creatine which has not reacted is then filtered off.

The volume of the filtrate is then carefully measured and the inorganic phosphate determined (see Chapter 15). Add the calculated amount of $MgCl_2$ and NH_4OH to precipitate all of the inorganic phosphate as $MgNH_4PO_4$. Add a few drops of $CaCl_2$ solution to the filtrate to remove the rest of the inorganic phosphate. Filter. Then add 40 grams of crystalline $CaCl_2$ and stir until it dissolves. Add 3 volumes of cold 95% alcohol (neutralized before use if necessary) with stirring and cooling. Let the mixture stand for 30 minutes in the cold. Centrifuge off the precipitate. Wash it with a small amount of cold water (to remove NaCl) and then with cold alcohol. Dry. This preparation can be used for enzyme studies although it contains considerable NaCl. It is however, easily obtained analytically pure by the treatment of Fiske and Subbarow (1929).

The preparation above is dissolved in water so that the solution contains 0.4 mg. total phosphorus per ml. Add an equal volume of filtered, saturated, $Ca(OH)_2$. The precipitate (inorganic phosphate and calcium carbonate) is filtered off. The filtrate is cooled to 0°C. with the least possible exposure to air and made just acid to brom-cresol-purple with dilute HCl. Three volumes of cold alcohol are added with stirring. After 30 minutes in the cold, the precipitate is filtered with suction, washed with alcohol and dried in air. The pure material has the composition: $C_4H_8O_5N_3PCa \cdot 4H_2O$ and should give 9.66% P on analysis. A second reprecipitation may be necessary. The yield is about 1-2 grams.

For use in enzyme studies the calcium can be removed by precipitation as the oxalate, the carbonate, etc. In all manipulations it must be kept in mind that the compound is extremely labile in acid solution (25% decomposition per hour at 20°C.) but is quite stable in alkaline solution. Its determination in tissue extracts is described in Chapter 15.

ADENOSINE-TRI-PHOSPHATE

G. A. LePage

It was found in this laboratory (Dubois, Albaum and Potter, 1943) that magnesium anaesthesia minimizes the breakdown of ATP which ordinarily occurs when the animals are killed by decapitation without anesthesia. The magnesium solution was made to contain 25% $MgSO_4$ (51% $MgSO_4 \cdot 7H_2O$), and was injected intraperitoneally. By giving a series of small injections the animal can be completely paralyzed and finally anaesthetized without respiratory failure, with the muscles remaining completely relaxed, and with no cyanosis. It is advisable to give about 250 mg. per kilogram followed by 125 mg. per kilogram every 10 minutes until complete anaesthesia is attained. The animal can usually be killed about 1/2 hour after the first injection. The muscles are removed from the back and the hind legs and cooled in cracked ice until all have been removed and trimmed. The trimmed muscles are then weighed (about 500 grams) and ground in the cold room in the presence of an equal volume of ice-cold 10% trichloracetic acid in a Waring Blendor. The precipitated muscle proteins and fiber masses are separated from the filtrate by squeezing through cheese cloth, and the residue is re-extracted in the Waring Blendor with an equal volume of cold 5% trichloracetic acid, and again strained through cloth. The combined extracts are filtered through a Buchner filter and brought to pH 6.8 (bromthymol blue indicator) with 10 N NaOH. The remainder of the procedure is essentially that of Needham. The dibarium salt of the adenosine triphosphate, along with any adenosine diphosphate, hexosediphosphate, phosphoglyceric acid and inorganic phosphate, is precipitated by addition of an excess of barium acetate (3.0 ml. of 2 M $Ba(OAc)_2$ per 100 gm. muscle will assure an excess). The suspension is chilled in the refrigerator one-half hour, then the mixed barium salts are centrifuged and the supernatant discarded. The precipitate is dissolved in 0.2 N HNO_3 (30-50 ml. per 100 gm. muscle used). A small insoluble residue should be filtered or centrifuged off. The solution is treated with a mercuric nitrate solution (Lohmann's Reagent: 100 gm. $Hg(NO_3)_2 \cdot 8H_2O$ + 25 ml. H_2O + 25 ml. conc. HNO_3; add the HNO_3 to the salt before the water), using 0.6 - 1.0 ml. per 100 gms. muscle used. After chilling in the refrigerator (15 min.), the precipitated nucleotides are centrifuged out and suspended in a small volume of water. Hydrogen sulfide is passed in to decompose the mercury salts, and the resulting HgS centrifuged, washed with acidified water, and discarded. The combined supernatant and washings are aerated until the excess of H_2S has all been removed. The solution is then neutralized to pH 6.8 and an excess of barium acetate added (1.0 ml. of 2 M $Ba(OAc)_2$ per 100 gm. of muscle will assure an excess here). The suspension is chilled one-half hour in the refrigerator, and the precipitate centrifuged. Successive washes of the precipitate are carried out with: (1) 1% $Ba(OAc)_2$ at pH 6.8 (6-8 times the volume of the wet precipitate); (2) 50% EtOH; (3) 75% EtOH; (4) 95% EtOH; (5) diethyl ether. The precipitate is dried in a desiccator over calcium chloride, preferably under vacuum.

Additional manipulations can be used to obtain a purer product. These involve the following: after the final barium ATP precipitate has been obtained, as above, before washing with alcohol and ether, one can dissolve it in 0.2 N HNO_3 and repeat the precipitation as mercury salt and conversion back to the barium salt. This gives a purer material. After centrifuging and even filtering off the HgS precipitate, one often finds a faint trace of HgS suspended in the solution. This contaminates the final product, and is undesirable. It can be removed before precipitating the Ba-ATP, while the solution is still acid, by addition to the solution of a small amount of $Ba(OAc)_2$ and 3-5 ml. of 0.1 N H_2SO_4 and centrifugation of the small $BaSO_4$ precipitate formed. This precipitate carries down with it the last traces of HgS.

Yields obtained from rabbits anaesthetized with $MgSO_4$ generally exceed 3.5 grams of the barium salt per kilogram of muscle. The material reprocessed as described contains 0.1-0.2% inorganic phosphorus, and is 98-99% pure calculated as the tetrahydrate ($Ba_2 \cdot ATP \cdot 4H_2O$). Two-thirds of the organic phosphorus is released as inorganic phosphorus on hydrolysis 7 minutes at 100°C. in 1 N HCl.

ADENOSINE-DI-PHOSPHATE

G. A. LePage

The method we have employed is modified from that of Lohmann and Shuster (1935), as follows:

A live lobster was cut in half, the tail muscles removed and 32 gm. of muscle weighed out. This was cut into small strips with a scissors and suspended in 300 ml. of chilled 0.45% KCl. After gentle agitation for 15 minutes, the muscle was filtered off and the supernatant discarded. The washing with 300 ml. portions of chilled 0.45% KCl solution

was repeated four times. The muscle was then suspended in 100 ml. of a solution containing 1.0 gm. of adenosine triphosphate at pH 7.0 and incubated for 20 minutes in another 100 ml. of 1% ATP. The muscle was filtered off and the easily (7 min., 1 N HCl, 100°C.) hydrolyzable phosphorus determined. This had decreased 52%.

The two solutions (freed of muscle) were mixed, adjusted to pH 6.8, and precipitated with an excess of 1 M barium acetate. After leaving it in the refrigerator one-half hour, the suspension was centrifuged and the supernatant discarded. The precipitate was dissolved in 0.2 N HNO_3 (solution kept cold) and treated with Lohmann's reagent (see preparation of ATP). The precipitate, after chilling for 15 minutes was centrifuged out and the supernatant discarded. The mercury precipitate was suspended in 150 ml. of water and decomposed with hydrogen sulphide. The HgS was centrifuged out, washed, and solution and washings aerated free of H_2S. Final traces of HgS, which tend to be colloidal, can be removed by the addition of barium, and a few ml. of 0.1 N H_2SO_4 (centrifuge out the $BaSO_4$). This carries down the last of the HgS. The solution was adjusted to pH 6.8, one volume of 95% EtOH and an excess of barium acetate added. This precipitates the pure barium salt of adenosine diphosphate. (Omitting the alcohol greatly reduces the yield because BaADP is much more soluble in pure solution than when inorganic phosphates are present). The precipitate was washed successively with 100 ml. portions of 50% EtOH (neutralized), 75% EtOH, 95% EtOH, and diethyl ether. It was then dried in a desiccator under vacuum, with $CaCl_2$.

Assuming the same water of crystallization as the ATP (4 H_2O), the product was 98% Ba·ADP·4 H_2O. The ratio of total organic phosphorus to easily hydrolyzable phosphorus was 2.03:1.00. Inorganic phosphorus was 0.31%.

ADENYLIC ACID

Two types of adenylic acid are available. The muscle adenylic acid (which contains the phosphate in the 5 position on the ribose) has the structure present in animal, yeast and most bacterial ATP (LePage and Umbreit, 1943b), and is therefore the one to be employed in most metabolic experiments. It is obtainable from the B. L. Lempke Co. (248 W. Broadway, New York City).

Yeast adenylic acid (containing the phosphate in the 3 position) is available from Lempke and may be readily isolated from yeast nucleic acid (Eastman) by the method of Jones and Perkins (1925). So far as is known there is only one ATP in which this structure occurs (LePage and Umbreit, 1943b). The two acids may be distinguished from one another by means of the hydrolysis rate of the ribose phosphates (LePage and Umbreit, 1943b) and by means of the test described by Klimek and Parnas (1932).

ADENOSINE

This compound is available from the B. L. Lempke Company.

ADENINE

This material is available from most chemical supply houses including the Eastman Kodak Company (Chemical Sales Division, Rochester, N. Y.).

INOSINE TRIPHOSPHATE

A. L. Lehninger

This compound is best made by deamination of adenosine triphosphate with nitrous acid according to Kleinzeller (1942).

The preparation of ATP has been described elsewhere (page 181) and a fairly pure preparation should be used. Two grams of Ba-ATP are dissolved in 0.1 N HCl, the Ba removed with Na_2SO_4, and the filtrate neutralized and brought to a volume of 32 ml. To this solution are added 4.52 gm. $CH_3COONa \cdot 3H_2O$, 10 ml. glacial acetic acid, and 20 ml. 60% $NaNO_2$. The pH of the solution should be about 4.0. After standing 5-6 hours at room temperature, cool with ice and add 2N NaOH until the pH is between 6.0 and 6.5. Add 12 ml. 3N $BaCl_2$ and complete the precipitation of the Ba salt of ITP by adding 2 volumes of cold alcohol. Centrifuge off the precipitate and suspend it in 50 ml. H_2O. Add 3N HCl

to the ice-cold suspension to bring the final concentration of HCl to 0.1 N. Shake the solution persistently until no more of the precipitate dissolves. Centrifuge off the precipitate and re-extract it with 20 ml. 0.1 N HCl. Add 1 gm. of urea to the combined centrifugates to destroy the remaining HNO_2. Keep at 0°C. for 2 hours. Then add 25 ml. 25% barium acetate and then a volume of alcohol equal to the total volume of solution plus barium acetate. Centrifuge off the precipitate, redissolve in 0.1 N HNO_3, and precipitate the ITP with Lohmann's reagent as is done in the preparation of ATP. The Hg compound is washed once with water, suspended in 50 ml. water and the Hg removed by passing H_2S through the solution. The HgS is removed by centrifuging, washed, and the combined centrifugates are aerated to remove H_2S. The solution is made 0.1N in HCl and the Ba salt precipitated as above (excess Ba acetate plus one volume alcohol). Redissolve and reprecipitate. Wash twice with water, twice with 50% alcohol, twice with absolute alcohol and once with ether. Dry in vacuo over $CaCl_2$. The yield is approximately 800 milligrams. The purity of the compound can be established by methods used for ATP. The empirical formula for the product is $C_{10}H_{10}O_4N_4P_3Ba_2 \cdot 7H_2O$ calculated % 7'P = 6.8%, total P = 10.3%, N = 6.20%. Ratio 7'P/total P = 0.667. The product will be almost completely pure, by these standards.

For use in enzyme experiments, the compound is converted into the sodium salt in the same way as is ATP. It is determined in an identical manner, i.e., by the phosphorus liberated by 7 minute hydrolysis in 1.0 N HCl at 100°C.

INOSINE DIPHOSPHATE

A. L. Lehninger

This compound is prepared by enzymatic hydrolysis (by a myosin preparation) of inosine triphosphate. The methods have been outlined by Bailey (1942) and Kleinzeller (1942).

The myosin or lobster muscle extract is prepared as in the section dealing with the preparation of adenosine diphosphate (page 181). The incubation of inosine triphosphate with the extract is carried out exactly as described there. To isolate the product from 1 gm. of the Ba-ITP, the myosin is removed by acidifying the reaction medium to pH 5.0 and centrifuging off the precipitate. The cooled supernatant is brought to pH 6.0 with N NaOH and 2-3 ml. of 25% barium acetate added. The resulting precipitate is centrifuged off and discarded. The supernatant is brought to pH 7.0 and the IDP precipitated with excess 25% barium acetate and an equal volume of cold alcohol. The precipitate is separated and suspended in H_2O. 3N HNO_3 is added to make a final concentration of 0.1N and excess barium acetate and an equal volume of alcohol added as before to precipitate the Ba-IDP. The compound is treated with Lohmann's reagent (see preparation of ITP, ATP) and after removal of the Hg with H_2S the filtrate is again adjusted to 0.1 N HNO_3, precipitated twice with barium acetate and alcohol as before. The product is washed with 50% alcohol until the washings are free of Ba (test with Na_2SO_4). It is then washed with 75%, then 95% alcohol, and ether. It is dried over $CaCl_2$ in vacuo. The yield is about 50-60% of theory. The product is very nearly pure. Calculated for $C_{10}H_{11}O_{11}N_4P_2Ba_{1.5} \cdot 6H_2O$; 7 min. P,4.20%; total P,8.39%; N,7.05%. Ratio 7 min.P to total P = 0.50.

The compound is converted to the soluble sodium salt by treatment with Na_2SO_4 in acid solution.

INOSINIC ACID

A. L. Lehninger

The method of Ostern (1932) has been found practical for the isolation of inosinic acid as the barium salt from fresh muscle tissue. One kilogram of fresh muscle (rabbit muscle has been used) is ground fine with a meat grinder and the ground tissue allowed to stand at room temperature for 3 hours to allow maximal enzymatic deamination of adenylic acid to inosinic acid. It is then mixed with a liter of water and slowly heated to boiling with constant stirring. Two ml. of glacial acetic acid are added to the mixture and it is boiled another three minutes. The mixture is then neutralized with NaOH and then made slightly acid with acetic acid, filtered through a fluted paper, and the residue pressed free of liquid. An excess of hot saturated $Ba(OH)_2$ solution is added to the filtrate to precipitate the inorganic phosphate and the mixture is allowed to stand for several hours. After testing for completeness of precipitation of inorganic phosphate

by $Ba(OH)_2$, the supernatant is decanted and the residue centrifuged. The precipitate is washed with a few ml. of warm water and the washings combined with the supernatant. The combined solutions are neutralized with glacial acetic acid and saturated aqueous lead acetate solution added to precipitate the nucleotide. Do not add a great excess of the reagent. After standing several hours the precipitate is filtered off and washed. It is then suspended in 300 ml. water and decomposed with H_2S. The precipitate (PbS) is thoroughly washed with warm water saturated with H_2S to remove the considerable amount of nucleotide adsorbed on the PbS. Powdered $BaCO_3$ is added to the combined filtrate and washings in excess, the solution brought to a boil, filtered hot, and concentrated in vacuo at 40-50°C to about 50 ml. The solution is filtered and further concentrated to a volume of about 10 ml. A white precipitate forms during the concentration. After the concentration is complete, the mixture is put at 0°C. for two days. The crystalline precipitate is filtered off cold and washed with a few ml. cold water. It is then dissolved in a minimum volume of boiling water, filtered hot, and allowed to crystallize at 0°C. The crystals are filtered off and washed with cold water. The yield is about 400-500 mg. pure barium inosinate. It is converted into the soluble sodium salt by removal of the Ba with Na_2SO_4.

PHOSPHOGLUCONIC ACID

W. W. Umbreit

This compound is readily prepared from either Embden's ester or glucose-6-phosphate by the method of Robison and King (1931). Using Embden's ester we have employed the following method:

Embden's ester (0.5 gm.), barium carbonate (0.6 gm.) and water (4 ml.) are ground together. Bromine (0.07 ml.) is added and the mixture stoppered and allowed to stand (30°C.) for 24 hours. The same quantity of bromine is again added and the mixture incubated for another 24 hour period. The excess bromine is removed by aeration, the mixture neutralized to pH 4 (to bring all of the barium carbonate into solution). The mixture is then fractionated according to the analytical procedure (Chapter 15), the barium insoluble fraction containing the phosphogluconic acid and inorganic phosphate. The barium is removed, the phosphate precipitated with magnesia mixture (see this page), and the phosphogluconic acid precipitated with barium. Yield; 200 mg. (Ba salt). By redissolving and reprecipitating the barium salt (dissolve in acid, reprecipitate at pH 8.2) an analytically pure product is easily obtained.

ACETYL PHOSPHATE

The synthesis of this compound has been described in detail by Lipmann and Tuttle (1944).

MAGNESIA MIXTURE

G. A. LePage

Preparation: Dissolve 55 gm. of $MgCl_2 \cdot 6H_2O$ and 100 gm. of NH_4Cl in 500-600 ml. of water. Add 100 ml. of 15M NH_4OH and make up to a liter. If the resulting solution is turbid, it should be filtered.

Conditions: This reagent is used in many instances for removing inorganic (ortho) phosphate from solutions of phosphate esters, etc. The desired product is $MgNH_4PO_4 \cdot 6H_2O$. If conditions are not correctly adjusted, the precipitate obtained may be $Mg(OH)_2$ or $Mg_3(PO_4)_2$. The solution is made alkaline to phenolphthalein with NH_4OH, an excess of magnesia mixture added, and sufficient NH_4OH added to make the solution 1.5 M with respect to NH_4OH. If stability of the other materials permits, (esters, etc.) the solution should be left 4 hours at room temperature to obtain quantitative precipitation of the inorganic phosphate as $MgNH_4PO_4 \cdot 6H_2O$.

Since the three phosphate radicles exist in equilibrium in solution, the amount of each will depend on the pH. Here one wishes to favor the ion HPO_4^{--}, which is accomplished by the use of NH_4OH. The function of the NH_4Cl is to prevent the precipitation of the $Mg(OH)_2$.

PRINCIPLE INTERMEDIATES IN THE KREBS' "TRICARBOXYLIC-ACID CYCLE"

After a long and somewhat controversial history, the Krebs' cycle has become a component part of our thinking with respect to animal metabolism. The subject has been very comprehensively reviewed by Krebs (1943). So far as is known the cycle as such does not occur, at least without modification, in molds or bacteria studied and there is indeed some doubt that, in spite of the fact that its component acids occur widely in plants, the cycle itself is in operation in these organisms. Integration of this scheme into the general metabolic picture of animal metabolism has been attempted by Potter and Elvehjem (1938) and Potter (1944). The compounds listed below are the principle intermediates of the cycle. However, one cannot, of course, be sure that they are the actual intermediates, since rapid phosphorylation and dephosphorylation, for example, are entirely possible. At least these are the materials one puts into the reactions and these are the materials one again obtains. Analytical methods for these compounds are described in Chapters 10 and 14.

Material	Page
Pyruvic acid	185
Isocitric acid	185
cis-Aconitic acid	185
alphá-Ketoglutaric acid	186
Oxalacetic acid	187

The other components of the cycle, e.g., citric acid, succinic acid, fumaric acid, and malic acid, are readily obtainable commercially. Brief remarks on the use of these are contained in Chapter 9.

CRYSTALLINE SODIUM PYRUVATE

H. A. Lardy

Pyruvate solutions for use in tissue respiration experiments are usually prepared by suitable dilution and neutralization of pyruvic acid redistilled at reduced pressure and stored in a cold place. A more convenient means of preparing pyruvate solutions for use as a substrate is by weighing out crystalline sodium pyruvate. This salt may be prepared analytically pure from commercial pyruvic acid by the procedure of Robertson (1942).

Commercial pyruvic acid is dissolved in ten volumes of ethyl alcohol. This solution is slowly neutralized with a solution of 1 volume saturated NaOH in 10 volumes of ethyl alcohol at room temperature. Sodium pyruvate crystallizes out immediately. The crystalline material is filtered off, washed with alcohol and ether on the filter and dried in a vacuum desiccator. The yield is about 85% of theory. This material is sufficiently pure for most purposes. Analytically pure salt may be obtained by recrystallization of the above preparation from 80% ethyl alcohol.

The potassium salt may be prepared in the same manner with somewhat better yields.

See also Chapter 9 for other purification methods.

ISOCITRIC ACID

This compound has been isolated by Pucher (1942) but is not otherwise available. In metabolic experiments it can usually be replaced by cis-aconitic acid since the two are in equilibrium in most tissues.

CIS-ACONITIC ACID

H. A. Lardy

Trans-aconitic acid is readily synthesized by the method of Bruce (1937). Cis-aconitic acid may be prepared from it by the method of Malachowski and Maslowski (1928) as follows:

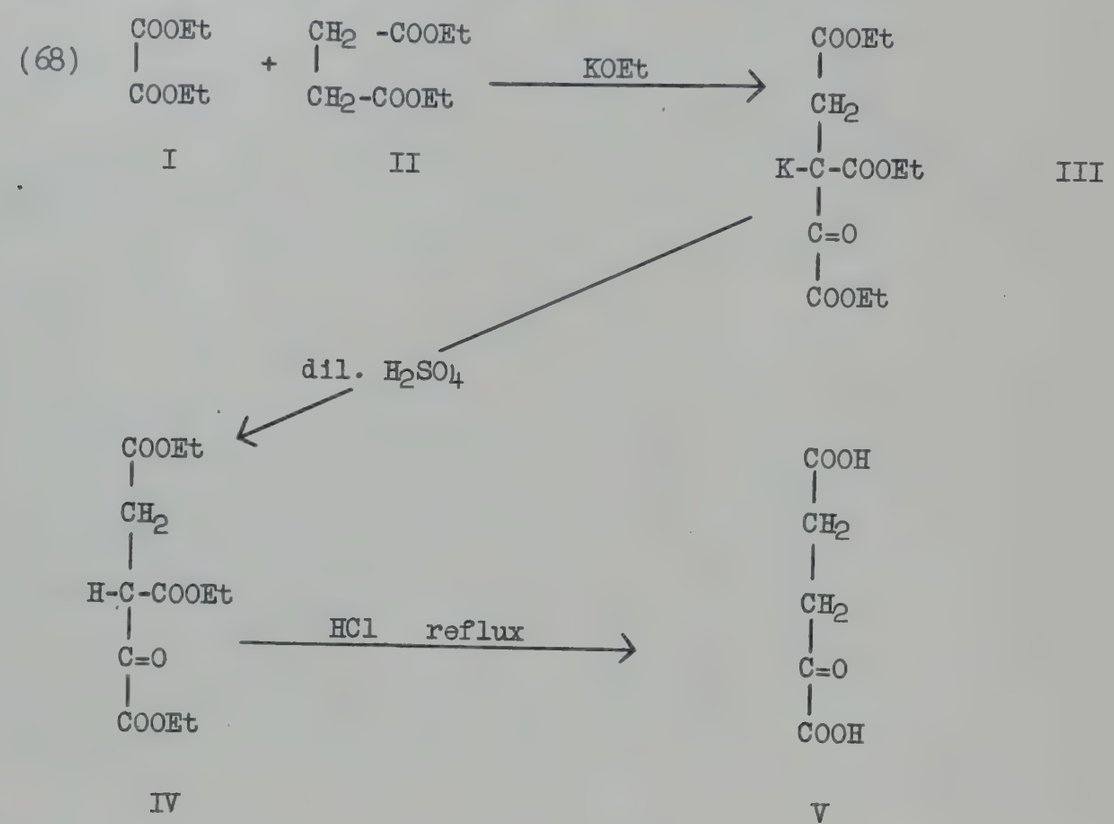
Recrystallize trans-aconitic acid from ether and conc. HCl until the melting point is at least 182°. Pulverize finely and mix with an equal weight of acetic anhydride and keep at room temperature for three days. The residue is filtered off, washed twice with

a 1:3 mixture of acetylchloride and chloroform and once with a small quantity of chloroform. The solid material is trans-aconitic anhydride. The filtrate and washings are combined and evaporated at room temperature under reduced pressure until the acetic acid has been removed. When dry, extract with boiling benzene and filter hot. Crystals of cis-aconitic anhydride with 1/2 mole of benzene form on cooling. The benzene is lost when the product is dried and the anhydride melts at about 74°. Further purification can be accomplished by recrystallizing from benzene. The anhydride has been found to be stable for two years and immediately forms cis-aconitic acid in water.

ALPHA-KETOGLUTARIC ACID

W. C. Schneider

This compound has been synthesized with the advice of Dr. P. P. Cohen. An essential part of the procedure is the use of the potassium salts. Sodium does not replace potassium in this case. The method for the synthesis is based on the papers by Wisclecenus and Waldmuller (1911) and by Neuberg and Ringer (1915). The type reaction is the acetoacetic ester condensation and the steps are as follows (68):



The necessary materials are as follows: 10.1 gm. potassium, 30.2 gm. absolute ethyl alcohol, 37.8 gm. ethyl oxalate, 45 gm. ethyl succinate, and 500 ml. dry ether. The first step is the preparation of potassium ethoxide, KOEt. This is done in a three necked flask fitted with a separatory funnel, a reflux condenser, and a mercury seal mechanical stirrer. The alcohol is added to the ether in the flask and then the potassium is introduced in the form of small pellets. The mixture is warmed slightly and complete solution of the potassium occurs after about two hours. The mixture is cooled in an ice bath and the ethyl oxalate (I) is added. Next, the ethyl succinate (II) is added; the solution turns lemon yellow and after about 15 minutes starts to solidify. When solidification begins, the ice bath is removed and the reaction allowed to continue for several hours. Before stopping the stirring, the mixture is heated to 40°C. for 15 minutes and then cooled.

The mixture is transferred to a Buchner funnel and washed with portions of dry ether until the filtrate is colorless. The residue, which is the potassium oxal-succinic acid ester is dissolved in water and the solution covered with a layer of ether and acidified to Congo Red with dilute sulfuric acid. The solution is extracted several times with ether. The combined ether extracts are neutralized with $BaCO_3$ and dried with anhydrous Na_2SO_4. The solution is filtered and the ether removed *in vacuo*. A thick bright yellow fluid, oxalsuccinic acid ester, remains. This is placed in a round bottom flask with 2 parts of concentrated HCl and 4 parts H_2O and refluxed 5-6 hours to hydrolyze the ester

to the free acid (V). This solution is extracted with ether. The ether is removed *in vacuo* and the white solid residue dried over P_2O_5 and soda lime. This is the α-keto glutaric acid, contaminated with about 5 per cent oxalic acid. The latter can be removed by dissolving in water and adding sufficient $CaCl_2$ solution to precipitate the oxalate as CaC_2O_4, which is filtered off. The α-ketoglutaric acid is recovered from the filtrate by extracting with ether and evaporating off the ether from the extract. The resultant product may be recrystallized by dissolving in acetone and adding excess benzene. The product is dried over P_2O_5 and stored in the cold over P_2O_5. Yield: 10 gms. or 26% of theoretical. Melting point: 112-113°C; 112°C. reported in literature.

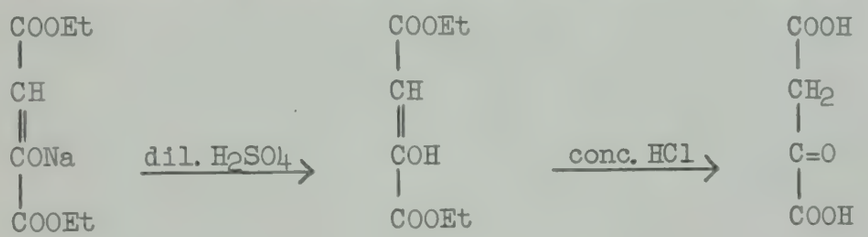
For use, a fresh portion of the acid is neutralized just before the experiment. The acid is dissolved in water to make 0.05 to 0.10 M and the theoretical amount of sodium bicarbonate solution is added with stirring. The evolved CO_2 will keep the solution acid until it is added to the reaction mixture, at which time the CO_2 absorber will take up any remaining CO_2 and the substrate will not alter the pH of the medium.

OXALACETIC ACID

W. C. Schneider

The preparation of this acid is considerably simpler than that of α-keto glutaric acid since sodium diethyloxalacetate is available commercially (Eastman, or U. S. Industrial Chemicals, Inc.), and one has only to do the hydrolyses and purification (Simon, 1903; Cohen, 1940; Krampitz and Werkman, 1941). The reactions are as follows (69):

(69)



I II III

100 gms. of sodium diethyl oxalacetate (I) are dissolved in the minimum amount of cold water and the solution covered with ether. The solution is acidified to Congo Red and extracted several times with ether. The ether extracts are neutralized with a small amount of $BaCO_3$, and dehydrated with Na_2SO_4 and then evaporated *in vacuo*. The liquid diethyl oxalacetate (II) remains; to it are added 4 parts of concentrated HCl and the mixture left to hydrolyze at 0°C. for 48 hours. The oxalacetic acid separates out in white crystals which are filtered off. Oxalacetic ester can be recovered from the HCl filtrate by ether extraction and re-treated with 4 parts of concentrated HCl to produce more oxalacetic acid. In this manner the yields of oxalacetic acid can be considerably improved. The oxalacetic acid separating from the HCl solution is dried over P_2O_5 and recrystallized by dissolving in acetone and adding excess benzene. The final product is a white powder and is stored in the cold over P_2O_5. Yield: 8.2 gms. or 9% of theoretical. Melting point: 150-152°C; 152°C reported.

Because of the lability of this acid, it is imperative that all operations, except those specified, be conducted in the cold.

For use, the oxalacetic acid is dissolved in water and the theoretical amount of sodium bicarbonate solution is added with stirring. This operation is carried out *immediately* before the substrate is added to the flasks, and the CO_2 absorbers take out the final CO_2. The solution is never stored.

IMPORTANT ELECTRON TRANSPORTING SYSTEMS AND CERTAIN COFACTORS

The materials of widest use are cytochrome c, and coenzyme I (diphosphopyridine nucleotide, cozymase). Both of these must be isolated from natural sources. A description of these and other electron (or hydrogen) transporting systems is given by Potter (1940).

While it is not our purpose to describe the preparation of enzymes certain of these have already been described in connection with other discussions. In addition, the preparation of "acetone powders" as a source of the enzymes, especially of the Meyerhof-Embden system is described below and the preparation of a common type of "Kochsaft" for supplying some of the cofactors in a relatively crude form is described. The preparation of enzymes and their study is described by Green (1940), Sumner and Somers (1943), and in the larger "Handbooks".

Normally the preparation of the active system one wishes to study is the responsibility of the individual investigator and is usually found in the literature of the subject with which he is concerned.

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PREPARATION AND STANDARDIZATION OF CYTOCHROME C

V. R. Potter

The method employed is essentially that of Keilin and Hartree (1937).

"One ox heart is carefully freed from fat and ligaments and minced very finely with a Latapie mincer. After pressing out the blood as far as possible in a handpress, the pulp, 1100 gm., is mixed with 1100 c.c. 0.15N* (2 1/2%) trichloracetic acid and allowed to stand at room temperature for 2 hours with occasional stirring. The pH of this mixture is approximately 4. The fluid is pressed out, neutralized to about pH 7 with caustic soda, and centrifuged for 10 minutes. The clear fluid, 1330 c.c. so obtained shows strong absorption bands of reduced cytochrome together with weak oxyhaemoglobin bands. It is treated with ammonium sulphate (50 g. per 100 c.c.), filtered, and the filtrate, 1700 c.c., now free from haemoglobin, is treated again with ammonium sulphate (5 g. per 100 c.c.) and left overnight in an ice chest. The pH of the mixture is about 4.9. Next day the liquid is filtered and, while still cold, treated with one-fortieth of its volume of 20% trichloracetic acid, bringing the pH of the mixture to 3.7. Within 10 minutes the spectrum of reduced cytochrome disappears and the cytochrome is completely precipitated in the oxidized form. The suspension is centrifuged for 10 minutes, the bright red deposit shaken with 500 c.c. saturated ammonium sulphate solution and centrifuged again. The red solid is transferred to a cellophane sac by means of about 20 c.c. distilled water and the mixture dialysed for 2 days at 4°C. against 1% sodium chloride solution. The content of the sac is shaken with a few drops of chloroform and filtered to yield about 30 c.c. of a clear dark red solution containing 0.182 g. pure cytochrome, the iron content of which is 0.34%."

In using this method we have dialysed against distilled water instead of 1% NaCl, in order to avoid having a compulsory addition of sodium chloride to our reaction mixtures. Since our cytochrome solutions keep very well in the cold, we have avoided the use of chloroform in the preparation. Keilin and Hartree also carried out large scale operations using about 6 beef hearts at a time, and pointed out that horse hearts are superior to beef hearts. For large-scale preparations it is advisable to use a power-driven meat grinder with a fine mince attachment. We have been able to secure the cooperation of a local butcher shop for the grinding operation.

The following table summarizes the results obtained in this laboratory in six consecutive preparations without particular attention to improvements in Keilin and Hartree's method (Table XXVII).

In terms of dry weight, the average yield is about 90 mg. per kg. of fresh beef heart. However the preparation is not dried, but is kept in the cold as a stock solution, from which working solutions can be prepared as needed. Keilin and Hartree in 1938 mentioned that cytochrome c can be precipitated by the addition of 4 volumes of cold acetone

*Checked by titration using phenolphthalein.

TABLE XXVII

The Preparation of Cytochrome C

No. of beef hearts	Kgm. of mince	ml. Stock Cytochrome Solution	Concentration per ml. Moles x 10^{-7}	Total Yield Moles x 10^{-7}	Yield per Kg Moles x 10^{-7}
5	6.69	160	2.19	350	52.4
6	6.43	151	2.28	344	53.6
6	6.99	143	3.03	433	62.0
6	7.29	186	2.60	485	66.5
10	14.30	180	3.06	552	38.6
10	10.87	122	4.88	596	54.8

Improvements communicated to the authors will be included in later editions.

and dried to a powder which is completely soluble in water. This seems to be true only when the solutions are kept very cold and the drying is very rapid, and we have avoided the procedure. The preparation has an equivalent weight of 16,500 and apparently contains an additional protein which can be removed to yield cytochrome with an apparent molecular weight of 11,700 (Theorell and Akesson, 1941). The possible catalytic action of this protein should be borne in mind when using the Keilin and Hartree preparation.

It is convenient to dilute the stock solution to a strength of 1.0 x 10^{-7} moles per ml., that is, 10^{-4} molar. In the succinoxidase system for example, one adds 4 x 10^{-8} moles of cytochrome c per Warburg flask, which is therefore 0.4 ml. of the working solution. The average preparation from six beef hearts thus gives enough cytochrome for about 1000 Warburg flasks when the succinoxidase system is being studied. In the cytochrome oxidase system, much higher concentrations are needed, and concentrated stock solutions are used directly. In this case it is convenient to recover the cytochrome. The flask contents are pooled, frozen, and stored until needed. The solution is then brought to pH 3.5 - 4.0 with trichloracetic acid, and carried through the regular procedure. Recoveries of around 95 per cent have been obtained.

In the final dialysis step of the purification, it is desirable to put the preparation through several changes of glass-redistilled water, to cut down the concentration of metal contaminants. This is especially important in the study of the cytochrome oxidase system. As the dialysis proceeds a dark brown precipitate forms, which may be denatured cytochrome c. This is removed by centrifugation and filtration and is discarded; the prevention of its formation has not been studied. Although Keilin and Hartree state that cellophane strongly adsorbs cytochrome c during dialysis in the absence of salts and Theorell used adsorption on cellophane at one stage of purification, we have not felt that the losses outweighed the advantages of having a pure aqueous solution.

There seems to be a certain amount of loss of cytochrome through the walls of the membrane, especially in the final stages of dialysis. This can amount to as much as 20 per cent of the yield and can be observed when the dialysis is carried out against relatively small volumes of distilled water. The cellophane is seamless tubing and is doubly knotted at each end, and tied between the knots.

Standardization of cytochrome c: For the most accurate work it is necessary to know the concentration of cytochrome c in the stock solution so that the proper amounts can be added in standardized experiments. Many experiments have been reported with no statement of the cytochrome c concentration; this is inexcusable. Even when the concentration of the cytochrome is unknown, it is possible to determine the amount which is necessary to saturate the system and to prove that additional amounts do not increase the reaction rate. Such data can then be included in the report.

Spectrophotometric Standardization: For more precise work, the spectrophotometric standardization seems the most satisfactory. Cytochrome c has a characteristic absorption spectrum in the oxidized state and an equally definite absorption in the reduced state (Theorell, 1936; Potter, 1941a). The reduced form has a pronounced maximum at 550 millimicrons, which is absent in the oxidized spectrum. The cytochrome c stock solution is a mixture of oxidized and reduced cytochrome c and must be converted to one form or the other before making any measurements for spectrophotometric standardization. The cytochrome can be oxidized with potassium ferricyanide and reduced with sodium hydrosulfite ($Na_2S_2O_4$). The hydrosulfite can be added after the ferricyanide to convert the oxidized form to the reduced form. Since the specific absorption coefficients are known for each form, one can measure the absorption at 550 mμ. in both oxidized and reduced states, and the concentration can be calculated for each. If the cytochrome solution is free from other pigments the same concentration will be found in each calculation. If other pigments are present they will be unlikely to show the same shift in absorption when converted from the oxidized to the reduced state. Neither the ferricyanide nor the hydrosulfite absorb light at 550 millimicrons. The pH of the mixture can be held constant with phosphate buffer. The constants are as follows:

$\alpha_R = 2.81(cm^2/mole) \times 10^7$ (Reduced Cytochrome c at 550 mμ.)

$\alpha_O = 0.90(cm^2/mole) \times 10^7$ (Oxidized cytochrome c at 550 mμ.)

These constants apply directly where the Cenco-Sheard Spectrophotelometer is used with the entrance slit at 0.7 mm. and the exit slit has a nominal width of 5 millimicrons and is not significantly different with an exit slit of 2.5 millimicrons. With exit slits wider than 5 millimicrons the constants do not apply. They should apply directly in the case of other spectrophotometers with similarly narrow exit slits. An example of a cytochrome c standardization is given herewith (Table XXVIII).

TABLE XXVIII

Spectrometric Standardization of Cytochrome c

Spectrophotometer Data at 550 mμ., S_1 = 0.7 mm.; S_2 = 5 mμ.
(Cenco-Sheard Spectrophotelometer)

Reaction Mixture		Io	I	Log I_o/I = E	Moles/ml. (cell)	Moles/ml. (Stock Solution)
Water	1.7 ml.					
0.1 M phosphate pH 7.4	1.0 ml.					
Stock Solution Cytochrome	0.2 ml.	=	oxidized state			
0.01 M $K_3Fe(CN)_6$	0.1 ml.					
final volume	3.0 ml.	94.2	62.0	0.181	0.201×10^{-7}	3.02×10^{-7}
Same solution plus						
0.1 to 1.0 mg. solid $Na_2S_2O_4$		=	reduced state			
		93.5	25.8	0.599	0.199×10^{-7}	2.98×10^{-7}

Calculation for oxidized cytochrome c:

The fundamental relation is $C = E/\alpha$; the cells are 1 cm. long.

$$C = E/\alpha = \frac{0.181}{0.90 \times 10^7} = 0.201 \times 10^{-7} \text{ moles per ml.}$$

There are 3.0 ml. in the reaction mixture, of which 0.2 ml. were stock solution.

Therefore, the stock solution contains

$$0.201 \times 3 \times 5 \times 10^{-7} = 3.02 \times 10^{-7} \text{ moles per ml.}$$

The calculations for the reduced cytochrome are the same, except α_R is used. The two values gave the same result within the limit of error and the average value was taken.

The fact that the ratio E_R/E_o is the same as α_R/α_o indicates that the cytochrome c solution is probably pure.

Standardization in Evelyn Colorimeter: If the cytochrome c is not contaminated with other pigments, it is possible to standardize the preparation on an Evelyn Colorimeter. In this case the light is not monochromatic and there is no point in trying to work with the narrow reduced absorption band at 550 mμ. Instead, the measurement is based on the broad absorption band of the oxidized form, which has its maximum at 530 mμ, but which may be measured on the Evelyn Colorimeter, using the 540 filter, and insuring complete oxidation by adding ferricyanide as in the spectrophotometric measurement. Dr. W. H. McShan has standardized this procedure using cytochrome c preparations whose concentration was determined by the author. The test mixtures contained 3.0 ml. 0.1 M phosphate buffer pH 7.4, 0.3 ml. 0.01 M $K_3Fe(CN)6$, plus varying amounts of Stock Solution of Cytochrome c whose concentration was 3.0×10^{-4} M, plus water to make 10.0 ml. The blank contained water instead of cytochrome, plus the other additions. The constant (K) was taken as 1.99×10^7 ml./mole. The data are given in Table XXIX.

TABLE XXIX

Data on Oxidized Cytochrome c. (Evelyn Colorimeter)

ml. Stock Cytochrome	R*	L**	Moles per ml. (Evelyn tube)	Moles per ml. (Stock Solution)
0.3	66.25	0.179	0.0900×10^{-7}	3.00×10^{-7}
0.4	57.75	0.238	0.1195×10^{-7}	2.99×10^{-7}
0.5	50.00	0.301	0.1512×10^{-7}	3.02×10^{-7}

* Galvanometer reading
** 2-log galvanometer reading

Calculation:

The fundamental relation is $C = \frac{L}{K}$ which corresponds to the relation $C = E/\alpha$ in the case of the spectrophotometric measurement. The calculations are made in a perfectly analogous manner. Thus, in the case of the first sample (0.3 ml.) the calculations are as follows:

$$C \text{ (Evelyn tube)} = \frac{L}{K} = \frac{0.179}{1.99 \times 10^7} = 0.090 \times 10^{-7} \text{ moles/ml.}$$

$$C \text{ (Stock Solution)} = 0.090 \times 10 \times 1/0.3 \times 10^{-7} = 3.00 \times 10^{-7} \text{ moles/ml.}$$

Enzymatic Standardization: Since the concentration of cytochrome c required to saturate a given enzyme system is a fundamental property of the system, a characteristic curve is obtained if one plots Q_{O_2} against cytochrome c molarity in the succinoxidase system, for example. In the case of this system the curve rises steeply and plateaus sharply at about 0.5×10^{-5} molar (Potter, 1941b). While this fact cannot be used to standardize cytochrome c solutions it provides excellent supporting evidence as to catalytic potency of a preparation.

DIPHOSPHOPYRIDINE NUCLEOTIDE (COENZYME I, COZYMASE)

A. L. Lehninger

A short method for the isolation of this coenzyme (claimed to be free from Coenzyme II) from bakers' yeast has been described by Williamson and Green (1940) and has been successfully used in this laboratory. To 2.8 liters of water at 90°C. are added 3.2 kg. of pressed starch free bakers' yeast. The yeast is crumbled and added slowly with constant stirring. The mixture is filtered hot with suction through a layer of kieselguhr (Filter-cel) supported on paper in a Buchner funnel. The filter is usually quickly clogged, hence 4 or 5 filter funnels are prepared and run simultaneously in order to complete the filtration while still hot.

To each 100 ml. of the clear filtrate add 12 ml. of 25% basic lead acetate. Filter through kieselguhr. The pH of the clear filtrate is then adjusted to 6.5, 35 ml. of 25% $AgNO_3$ added, and the precipitate allowed to settle. The supernatant solution is decanted off and the precipitate collected by centrifuging. It is washed three times with water and suspended in 50 ml. of water. The Ag is removed by H_2S. The H_2S is removed from the filtrate by aeration and the solution is poured into 5 volumes of cold acetone with stirring. After standing at 0°C for 2 hours, it is filtered quickly through a sintered glass funnel in the cold room and washed with cold acetone. The product is dried in vacuo over H_2SO_4. The yield is between 0.4 and 0.6 grams.

This product is at least 40% pure. Williamson and Green report a product of 65% purity using this method. The purity can be accurately determined spectrophotometrically or manometrically (Warburg, et al., 1935). For use in enzyme studies, the product is simply dissolved in water and neutralized.

PREPARATION OF "KOCHSAFT" (BOILED MUSCLE EXTRACT)

H. A. Lardy

There are perhaps as many ways of preparing muscle "kochsaft" as there are investigators who have used such a preparation. A procedure which has been found satisfactory in our laboratory is as follows:

Strips of muscle are rapidly removed from a freshly killed rat and dropped into boiling water. Five volumes of water to one of muscle is a suitable ratio. After the last strip added has boiled for 2 to 5 minutes the mixture is cooled. The solid material is thoroughly ground with mortar and pestle. Water is added to make up to the original volume and the mixture boiled again for a few minutes. The solids are then removed by centrifuging or filtering and the clear portion is stored in a frozen condition until used.

PREPARATION OF ACETONE POWDERS

V. R. Potter

The method of preparation is essentially that of Green, Needham, and Dewan (1937) as follows:

"The skeletal muscles of a freshly killed animal (rabbit) are cooled by packing with ice and thoroughly minced. The mince is mixed with 2 volumes of iced water and allowed to stand for ca. 30 min. The mixture is squeezed through muslin. 2 volumes of cold acetone are then added to the filtrate. The precipitate is filtered immediately on Buchner funnels with suction and then washed with acetone and ether. If the washings are effected before the cake of precipitate has cracked, it is possible to pulverize the precipitate in a mortar and dry it within an hour in vacuo over liquid paraffin. The dried acetone powder (10 g.) is rubbed up with water (120 ml.) until a homogenous paste is formed. The mixture is then dialysed for 15 hours at 0°C. in cellophane sacs. The large amount of insoluble material is centrifuged off and discarded. The clear supernatant fluid contains the active enzymes. The activity is maintained for at least 10 days if the enzyme solution is kept at 0°. The enzymes are best kept in the form of the dry powder."

In our experience, the final dehydration of the precipitated protein with pure acetone and ether, must be done quickly and in the cold. Furthermore in trying to suck the precipitate dry, one is likely to hydrate it simply by contact with air. When this occurs, the color of the precipitate changes to a dark brown, and the dried precipitate is dark in color instead of almost white. The dark precipitate contains much more water-insoluble and presumably denatured protein than is the case with the light colored powder which is obtained when the dehydration is properly carried out. We have used from 0.5 to 1.0 ml. of the enzyme solution per Warburg flask in studies involving the addition of dehydrogenases. Further work needs to be done on the fractionation of the enzymes in this preparation, and on the factors affecting their solubility and denaturation.

BUFFERS AND SUSPENDING MATERIALS

Certain solutions are in wide use and are more or less a part of the knowledge of the study of tissue metabolism. The formulae for the more common of these are here given, together with a brief discussion of suspending media and buffers which should help the beginner choose the proper conditions for operation.

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SUSPENDING MEDIA FOR ANIMAL TISSUES

P. P. Cohen

The choice of a suspending medium for a given tissue preparation is to a large measure determined by the nature of the metabolic experiment. In the case of tissue slices it is ordinarily assumed that the purpose of the medium is to provide a solution which because of its ionic composition and its osmotic relation to the cells will maintain the integrity of the latter. Thus, the commonly employed Krebs-Ringer solution (Krebs and Henseleit, 1932) is so constituted as to closely approximate the ionic composition of the mammalian serum, (see below). This medium would seem to provide a physiological extra-cellular environment and so insure the metabolic integrity of the surviving cells. While this may actually be the case, it would appear from the literature that optimum conditions for a given metabolic reaction with slices often require a medium which is different from the balanced, physiological salt solution. Thus, the synthesis of glycogen from pyruvate by liver slices is most rapid when a medium high in potassium is used (Buchanan et al., 1942). The medium the latter investigators found to give optimum glycogen formation consisted of (per liter) $CaCl_2$, 5.6 mM.; KCl, 75 mM.; K pyruvate, 60 mM; and $KHCO_3$, 43 mM. As can be seen, this medium can hardly be considered a balanced solution in the usual sense of the word. The respiration of brain tissue is markedly sensitive to variations in ionic composition of the suspending medium. When compared with other tissues such as kidney cortex, testis, liver, yolk sac, and retina, brain stands out as being unusually sensitive to the effects of changes in ionic concentration (Dickens and Greville, 1935).

Many other such instances of the effect of varying ionic concentrations on metabolic reactions with tissue slices have been reported. It is only necessary here to stress that the choice of a nutrient medium for a given metabolic experiment with tissue slices may require considerable experimentation with the composition of the medium before optimum conditions are realized.

The Use of Serum as a Suspending Medium: The use of mammalian serum in place of balanced salt solution would seem to represent the ideal physiological medium for suspending tissue slices. While some differences in O_2 and R. Q. have been reported (Dickens and Simer, 1931; Canzanelli and Rapport, 1939) the magnitude of the difference is usually not great enough to warrant its routine use. As a matter of fact, in some instances respiration is greater in salt solutions than in serum. Some of the difficulties attending the use of serum are:

1) Its preparation
2) The considerable CO_2 retention, and
3) The uncontrollable variability in its make up from sample to sample.

The latter would seem to be particularly undesirable since it represents an introduction of unknown variables in a system which has as one of its features the control of the tissue's environment.

Suspending Media for Tissue Minces: Since tissue minces contain a higher per cent of broken cells, the choice of a suspending medium would seem to favor one more closely approximating intra- rather than extracellular fluid. However, here again it is not possible to predict what type of medium will be most suitable for any given experiment. In the case of pigeon heart muscle mince, a phosphate-saline medium containing NaCl, KCl, $MgSO_4$ and Na_2HPO_4 was found to give higher metabolic rates than phosphate buffer plus NaCl, or phosphate buffer plus $MgCl_2$ (Krebs and Eggleston, 1940). A study of the effect

of electrolytes on the respiration of pigeon heart muscle mince has been reported by Kleinzeller (1940). It was found that the optimal K concentration was 0.0385 M if the medium contained 0.02 M phosphate and 0.0425 M NaCl. When the medium contained 0.02 M phosphate, 0.092 M NaCl and 0.00085 M $MgSO_4$, the optimal K concentration was 0.0034 M. The optimum concentration of Mg was dependent on the concentration of other ions in the medium. Thus in a medium containing "physiological" concentrations of Na, K, and Cl the optimal Mg concentration was 0.0025 M. With a K concentration of 0.0385 M , the optimal Mg concentration is about 0.00125 M.

Phosphate, CO_2-Bicarbonate and other buffers: From a quantitative standpoint the CO_2-bicarbonate system of the extracellular fluid is the chief buffer system in the body. In the case of tissue slices, the use of phosphate buffer is somewhat more common since it is easier to use. Dickens and Simer (1931) found no significant difference in the Q_{O_2} or RQ of tissue slices in bicarbonate (or phosphate) Ringer solutions. However, more recently, Laser (1942) has shown that in the absence of CO_2 the maximum activity of tissue slices and reactivity to substrates are maintained for only a short time. Further, it was found that CO_2 stabilized the Q_{O_2} of slices in the presence of substrate for several hours and also insured their ability to oxidize substrates added after an incubation period without substrate. Since it is customary to "gas" phosphate buffered systems with 100% O_2, it should be pointed out that Laser has demonstrated that the rate of O_2 uptake without added substrate declined more rapidly in 100% than in lower O_2 tensions.

The use of buffers other than bicarbonate and phosphate has received relatively little study. In a comparative study of various media buffered with phosphate, bicarbonate and borate, Feinstein and Stare (1940) found that with liver slices there was no essential difference in the O_2 uptake. On the other hand, minced liver showed a higher O_2 uptake with the borate buffer than with phosphate or bicarbonate.

Preparation of Krebs-Ringer-Phosphate and Bicarbonate Solutions:

Solutions:

1) 0.90% NaCl	(0.154 M)	
2) 1.15% KCl	(0.154 M)	
3) 1.22% $CaCl_2$	(0.11 M)	(5 ml. equivalent to 11 ml. 0.1N $AgNO_3$)
4) 2.11% KH_2PO_4	(0.154 M)	
5) 3.82% $MgSO_4 \cdot 7H_2O$	(0.154 M)	
6) 1.30% $NaHCO_3$	(0.154 M)	(gas with CO_2 for 1 hour)
7) 0.1 M phosphate buffer, pH 7.4		(17.8 g. $Na_2HPO_4 \cdot 2H_2O$ + 20 ml. 1N HCl; dilute to 1 L.)

To prepare the Krebs-Ringer solution, the following amounts of the above are mixed:

100 parts of solution 1)	Note: All solutions are isotonic with rat serum, hence can be mixed in any proportion yielding mixtures (of differing composition) which are still isotonic.
4 " " " 2)	
3 " " " 3)	
1 " " " 4)	
1 " " " 5)	

If Krebs-Ringer-Bicarbonate is desired, 21 parts of solution 6) are added. The solution is then gassed for 10 minutes with 5% CO_2. For aerobic experiments 5% CO_2-95% O_2 mixture is usually used, and for anaerobic experiments, 5% CO_2-95% N_2. After mixing and gassing the solution, it should be kept in a glass stoppered vessel in the cold, until ready for use. The manometric flasks containing the solution should be attached to the manometers and "gassed" as soon as possible.

If Krebs-Ringer-Phosphate is desired, 12 parts of solution 7) are added in place of the bicarbonate solution. This solution after mixing, is gassed with either O_2, H_2 or air, depending on the gas phase desired.

To simplify the preparation and handling of the above solutions, it has been found convenient to make up solutions 1) to 5) five times the concentrations listed. The more concentrated solutions are stable for months when stored in the cold. For a stock Krebs-Ringer solution, solutions 1) to 5) are made up in the proportions indicated above (taking

into account the concentration factor of 5). This will keep in the cold for about one week. To make up the Krebs-Ringer bicarbonate solution 10 ml. of solution 6) are diluted to 100 ml. with the stock Krebs-Ringer solution. To make up the Krebs-Ringer-Phosphate, 10 ml. of solution 7) are diluted to 100 ml. with the stock Krebs-Ringer solution.

BUFFERS

W. W. Umbreit

It is assumed that the reader is familiar with the concept of pH. We wish here merely to develop the "pKa" concept and to show its usefulness.

Any acid, capable of ionization, when placed in an aqueous solution would liberate hydrogen ions according to the following equation:

$$HA \rightleftharpoons H^+ A^-$$

or

$$(H^+)(A^-)/HA = K$$

If one takes the logarithms of both sides of the equation one has,

$$\log (H^+)(A^-)/HA = \log K$$

$$\log H^+ + \log A^- - \log HA = \log K$$

$$\log H^+ = \log K + \log HA - \log A^-$$

$$-\log H^+ = -\log K + \log A^-/HA$$

Under practical circumstances, any salt of the acid present will contribute A^- ions, hence

$$pH = -\log K + \left|\log \frac{salt}{acid}\right|$$

The term -log K may be expressed as pKa (Equation 70):

$$(70) \quad pH = pKa + \left|\log \frac{salt}{acid}\right|$$

It would thus appear from equation (70) that when the acid is half neutralized, the pH of the solution would be pKa. And when 10% of it were neutralized, roughly, (pH = pKa + log 1/10 = pKa -1) the pH would be 1 unit lower than the pKa; when 90% was neutralized, the pH is roughly 1 unit higher than the pKa. Over the range of physiological activity one can thus pick buffers which will maintain the pH relatively constant.

A base will dissociate as follows:

$$BOH \rightleftharpoons B^+ + OH^-$$

or $$K_b = \frac{(B^+)\ (OH^-)}{BOH}$$

taking the logarithm of both sides:

$$\log K_b = \log B^+ + \log OH^- - \log BOH$$

$$-\log OH^- = -\log K_b + \log \frac{B^+}{BOH}$$

$$= -\log K_b + \log \frac{salt}{base}$$

-log OH may be expressed as pOH.

But since the dissociation constant of water is 1×10^{-14},

$$pH = 14 - pOH, \qquad \text{hence } pOH = 14 - pH$$

$$14 - pH = -\log K_b + \log \frac{salt}{base}$$

$$pH = 14 + \log K_b - \log \frac{salt}{base}$$

$-\log K_b$ can be designated as pK_b, hence this equation may be expressed as:

$$pH = 14 - pK_b - \log \frac{salt}{base}, \text{or}$$

$$pOH = pK_b + \log \frac{salt}{base}$$

Acetate buffers:

$$K = 1.86 \times 10^{-5} \qquad pKa = 4.730$$

These buffers would work well over the range of 3.7 to 5.7 (representing 10 to 90% neutralization).

Phosphate buffers:

$$K_1 = 1.1 \times 10^{-2} \qquad pKa = 1.959$$

$$K_2 = 2 \times 10^{-7} \qquad pKa = 6.7$$

$$K_3 = 3.6 \times 10^{-13} \qquad pKa = 12.44$$

These can then cover the range from:

1 to 3	$H_3PO_4 \rightleftharpoons KH_2PO_4$
5.7 to 7.7	$KH_2PO_4 \rightleftharpoons K_2HPO_4$
11 to 13	$K_2HPO_4 \rightleftharpoons K_3PO_4$

The range of physiological interest is:

$$KH_2PO_4 \rightleftharpoons K_2HPO_4$$

Similar buffers:

Carbonic, $K_1 = 3 \times 10^{-7}$ $(H_2CO_3 \rightleftharpoons KHCO_3)$

Maleic, $K_2 = 2.6 \times 10^{-7}$

Borate buffers:

$$K_3 = 6.4 \times 10^{-10} \qquad pKa = 9.193$$

Range 8.2 to 10.2

Several "universal" buffers have been described. For example, that of Teorell and Stenhagen (1938) is made by mixing NaOH, H_3PO_4, citric acid, HCl and H_3BO_3. Over the range of pH 3 to 11, the buffer capacity is virtually linear and the addition of 1/20 volume 0.1N HCl or NaOH shifts the pH about 1 unit.

Bases as Buffers: Certain bases serve as buffers in physiological ranges. For example, ammonium hydroxide has a dissociation constant of 1.8×10^{-5} (K_b).

Thus $pK_b = -\log 1.8 \times 10^{-5} = 4.74$

Hence ammonia - ammonium salt mixtures will operate best over the pH range of 8.3 to 10.3 since;

$$14 - 3.74 = 10.3$$

$$14 - 5.74 = 8.3$$

In the case of bases, the lower the numerical value of the dissociation constant the lower the pH in which they are good buffers. For example, pyridine ($K_b = 2.19 \times 10^{-9}$) is best over the range 4.5 to 6, while ammonia 1.8×10^{-5} is best over the range 8.5 to 10.

The composition of the buffer is not to be disregarded in physiological experiments. If one prepares phosphate buffers, for example, by mixing the mono- and di-hydrogen potassium salts, not only does the pH change but also the amount of potassium. One must take care that the effects observed with the altering of pH are due to the hydrogen ion and not other alterations in the buffer.

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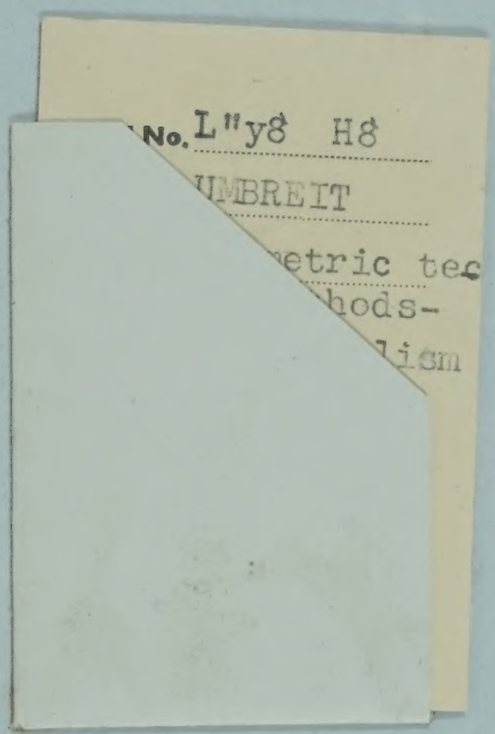
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